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Studies were conducted related to the development of small fish carcinogenesis bioassay models using the medaka (Oryzias latipes) and the guppy (Poecilia reticulata). The specific aim of the project was to identify certain classes of carcinogens that would induce neoplastic lesions in those model species. Bioassays were conducted on the following compounds: the halogenated hydrocarbons 1,1,2,2-tetrachloroethane (TeCE), chlorodibromomethane (CDBM), and vinylidene chloride (VDC), the heavy metal cadmium (Cd), the aromatic amines 2-acetylaminofluorene (AAF) and 4-aminobiphenyl (ABP), the nitrogen-substituted polyaromatic hydrocarbon dibenzocarbazole (DBC), the industrial product acrylonitrile (AN) and the pharmaceutical methapyrilene (MP). Bioassays were conducted on TeCE, CDBM, VDC, Cd, AAF, ABP, AN, and MP on medaka and guppy and DBC on medaka only. Mechanisms of ethylene dibromide metabolism were examined in medaka and of acetylaminofluorene in medaka and guppy. Other carcinogen bioassay factors such as the occurrence of background and rare carcinogen-induced tumors were examined in both species.

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19. Abstract (Continued)

We established that vinylidene chloride was carcinogenic to both the medaka and guppy, causing hepatic neoplasms in both species. Exposure to acetylaminofluorene resulted in the induction of hepatic neoplasms in both species but in higher incidences in the guppy than in the medaka. In studies of the metabolism of AAF by liver microsomes, the enhanced carcinogenicity of AAF in the guppy was related to the ability of the guppy to produce more of the N-hydroxylated metabolite, the carcinogenic metabolite, than the medaka which produced more of the ring-hydroxylated metabolites which result in detoxification. Studies on the hepatic metabolism of ethylene dibromide in the medaka indicated that the carcinogenic mechanism of this compound is similar in this species to that in rodents and may depend on a phase II (detoxification) pathway to exert its carcinogenicity. The other compounds were negative in our tests. We would prefer, however, to consider that these compounds showed no evidence of carcinogenicity in the fish models under the conditions of the tests rather than considering them non-carcinogenic in the test species.

Using the large histopathological database developed in this project, we have established the occurrence of spontaneous (background) neoplasms for the medaka and guppy. The rate of spontaneous hepatic neoplasms in designated control specimens was confirmed to be low, far less than one percent, in specimens examined at the critical sampling times of 24, 36, and 52 weeks. Other background neoplastic lesions that we identified in over 14,000 medaka examined histologically included thymic (lymphoblastic) lymphoma (about 25 cases), pancreatic acinar cell carcinoma (7 cases), and germ cell neoplasms (24 cases) resembling spermatocytic seminoma. Non-hepatic neoplasms occurring in the guppy from over 12,000 specimens include only a few individual cases including an adenocarcinoma of the retinal pigment epithelium and a pigment cell neoplasm, and a few cases of swim bladder epithelial neoplasms.

FOREWORD

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William E. [signature] 12-20-91

PI Signature Date

Table of Contents

i. Executive Summary

Studies were conducted related to the development of small fish carcinogenesis bioassay models using the medaka (*Oryzias latipes*) and the guppy (*Poecilia reticulata*). The specific aim of the project was to identify certain classes of carcinogens that would induce neoplastic lesions in those model species. Bioassays were conducted on the following compounds: the halogenated hydrocarbons 1,1,2,2-tetrachloroethane (TeCE), chlorodibromomethane (CDBM), and vinylidene chloride (VDC), the heavy metal cadmium (Cd), the aromatic amines 2-acetylaminofluorene (AAF) and 4-aminobiphenyl (ABP), the nitrogen-substituted polyaromatic hydrocarbon dibenzocarbazole (DBC), the industrial waste acrylonitrile (AN) and the pharmaceutical methapyrilene (MP). Bioassays were conducted on TeCE, CDBM, VDC, Cd, AAF, ABP, AN, and MP on medaka and guppy and DBC on medaka only. Mechanisms of ethylene dibromide metabolism were examined in medaka and of acetylaminofluorene in medaka and guppy. Other carcinogen bioassay factors such as the occurrence of background and rare carcinogen-induced tumors were examined in both species.

We established that vinylidene chloride was carcinogenic to both the medaka and guppy, causing hepatic neoplasms in both species. Exposure to acetylaminofluorene resulted in the induction of hepatic neoplasms in both species but in higher incidences in the guppy than in the medaka. In studies of the metabolism of AAF by liver microsomes, the enhanced carcinogenicity of AAF in the guppy was related to the ability of the guppy to produce more of the N-hydroxylated metabolite, the carcinogenic metabolite, than the medaka which produced more of the ring-hydroxylated metabolites which result in detoxification. Studies on the hepatic metabolism of ethylene dibromide in the medaka indicated that the carcinogenic mechanism of this compound is similar in this species to that in rodents and may depend on a phase II (detoxification) pathway to exert its carcinogenicity. Although the other compounds were negative in our tests, we would prefer at this time to consider that these compounds showed no evidence of carcinogenicity in the fish models under the conditions of the tests rather than considering them non-carcinogenic in the test species.

Using the large histopathological database developed in this project, we have established the occurrence of spontaneous (background) neoplasms for the medaka and guppy. The rate of spontaneous hepatic neoplasms in designated control specimens was confirmed to be low, far less than one percent, in specimens examined at the critical sampling times of 24, 36, and 52 weeks. Other background neoplastic lesions that we identified in over 14,000 medaka examined histologically included thymic (lymphoblastic) lymphoma (about 25 cases), pancreatic acinar cell carcinoma (7 cases), and germ cell neoplasms (24 cases) resembling spermatocytic seminoma. Non-hepatic neoplasms occurring in the guppy from over 12,000 specimens include only a few individual cases including an adenocarcinoma of the retinal pigment epithelium and a pigment cell neoplasm, and a few cases of swim bladder epithelial neoplasms.

1.0. Introduction

1.1. Overview of the project. This project was designed to facilitate the development of small fish carcinogenesis bioassay models, specifically the guppy and medaka, by identifying the classes of carcinogens to which the models respond and to gain insight on carcinogenic mechanisms in the two species. An ancillary aim of the project was to examine factors that affect the interpretation of the results of small fish carcinogenesis bioassays such as the occurrence of background and rare carcinogen-induced tumors. We conducted bioassays on the following compounds: the halogenated hydrocarbons 1,1,2,2-tetrachloroethane (TeCE), chlorodibromomethane (CDBM), and vinylidene chloride (VDC), the heavy metal cadmium (Cd), the aromatic amines 2-acetylaminofluorene (AAF) and 4-aminobiphenyl (ABP), the nitrogen-substituted polyaromatic hydrocarbon dibenzocarbazole (DBC), the industrial waste acrylonitrile (AN) and the pharmaceutical methapyrilene (MP). Bioassays were conducted on TeCE, CDBM, VDC, Cd, AAF, ABP, AN, and MP on medaka and guppy and DBC on medaka only. Exposures for bioassays of TeCE, CDBM, and VDC were conducted under flow-through conditions. For AAF, ABP, MP, and DBC, exposures were conducted under static or static renewal conditions. Carcinogenicity of Cd and AN were examined using both intraperitoneal injection and static exposures in separate tests. The bioassays are summarized in Table 1.1. Test numbers given are for cross-reference in the raw data package. Exposures to examine the carcinogenicity of ethylene dibromide (1,2-dibromoethane; DBE) were conducted under another project. In the present project, those results were analyzed and biochemical studies of DBE carcinogenesis in medaka were conducted. Studies on the metabolism of AAF related to carcinogenesis were conducted in medaka and guppy.

Confirmation of the diagnosis and documentation of the occurrence of background neoplasms are important in developing carcinogenesis bioassay models. In this project, we placed considerable emphasis on using our large and developing histopathological database to examine rarely-occurring neoplasms and distinguish between those that are spontaneous (background) and those that appear carcinogen-induced. Background neoplasms that have been analyzed in the medaka, which appears far more susceptible than the guppy to the development of background neoplasms, include thymic lymphoma, acinar cell carcinoma of the exocrine pancreas, germ cell neoplasms, and hepatocellular proliferative lesions. In the guppy, we analyzed hepatocellular proliferative lesions and a case of adenocarcinoma of the retinal pigment epithelium. Otherwise, the rate of occurrence of background neoplasms in the guppy is negligible.

Table 1.1. Summary of small fish carcinogenicity bioassays.

GCRL Test No.	Test Compound	Species	Type Exposure
FT 60	Tetrachloroethane	Medaka	Flow-through
FT 61	Tetrachloroethane	Guppy	Flow-through
FT 67	Vinylidene chloride	Medaka	Flow-through
FT 68	Vinylidene chloride	Guppy	Flow-through
FT 71	Chlorodibromomethane	Medaka	Flow-through
FT 72	Chlorodibromomethane	Guppy	Flow-through
PD 52	Cadmium	Medaka	Static/multiple
PD 54	Acetylaminofluorene	Medaka	Static/multiple
PD 55	Acetylaminofluorene	Guppy	Static/multiple
PD 61	Cadmium	Medaka	IP injection
PD 63	Cadmium	Guppy	IP injection
PD 65	Acrylonitrile	Medaka	IP injection
PD 66	Dibenzocarbazole	Medaka	Static/multiple
PD 73	Methapyrilene	Guppy	Static/multiple
PD 74	Methapyrilene	Medaka	Static/multiple
PD 75	Acrylonitrile	Guppy	Static/multiple
PD 76	Acrylonitrile	Medaka	Static/multiple
PD 77	Aminobiphenyl	Medaka	Static/multiple
PD 70	Aminobiphenyl	Medaka	Static/multiple
PD 71	Aminobiphenyl	Guppy	Static/multiple

1.2. Organization of the report. This report is presented as a series of individual studies in a format similar to that used for articles submitted for publication in the referenced literature as suggested in Section F.4b of the contract reporting instructions. Because of this type of format, there is some overlap in information. Because of the scope of this study and the concomitant mass of raw data accumulated, we have included the following: (1) complete raw data for one representative test, the CDBM flow-through exposure with medaka and guppy; (2) representative raw data from all other tests supported by this project;

and, (3) complete histopathology data for all specimens examined in the study. Printouts of histopathologic analyses are included for each test that was conducted. We will provide any other raw data or specific data packages that may be required. Otherwise, all raw data will be maintained at the Gulf Coast Research Laboratory for at least five years. Furthermore, histopathology data, paraffin blocks, and glass slides containing specimens from this study will be permanently archived.

We begin with a critical analysis of small fish carcinogenesis bioassays and describe test organization and rationale. The following chapter describes the general methods that were utilized in the study. Chapters dealing with analyses of test results make up the middle chapters. The latter chapters deal with analyses of background and spontaneous neoplasms in the medaka and guppy.

1.3. Acknowledgements. We wish to take this opportunity to thank the project officer, Mr. Henry S. Gardner, U.S. Army Medical Research and Development Command, Fort Detrick, for his support, guidance, and patience. During the study, he shouldered many administrative burdens that allowed us to devote our attention to the scientific portions of the project. We also wish to thank Ms. Nancy Mohler, Medical Research Acquisition Activity, for her cooperation and assistance with budgetary matters.

2.0. A critical analysis of the use of small fish in carcinogenicity testing

Rationale for the use of fish to detect chemical carcinogens. Toxicity tests using aquatic organisms now include such sophisticated endpoints as metabolic conversions of carcinogens and modifications of DNA (Malins and Ostrander, 1991). The objective of this chapter will be to provide examples of practical approaches to using small aquarium fishes to detect genotoxic chemicals or compounds in tests in which neoplasm development and associated pathological changes are the primary endpoints. Because the development of cancer following exposure to a carcinogenic material is a latent or chronic toxic response, a whole-animal carcinogenesis test using a fish model can be considered an elaboration or extension of an acute or chronic aquatic toxicity test.

The use of fish models for carcinogenesis research has received considerable attention in recent years (e.g., Black, 1984; Couch and Harshbarger, 1985; Dawe et al., 1981; Hawkins et al., 1988; Hendricks, 1982; Hoover, 1984; Masahito et al., 1988; Metcalfe, 1989; Mix, 1986; Powers, 1989). Small fish carcinogenesis models are being developed with several related applications in mind. Small fish could be used and, in some limited cases have been used, to examine the carcinogenicity of water-borne and sediment-bound compounds that have been implicated in the occurrence of cancer of the liver and other organs in wild fishes.

Another application of small fish carcinogenesis models concerns their potential for identifying and predicting health effects. Black (1984) and Metcalfe (1989) specifically dealt with tests to identify carcinogens. Because of several factors such as economy, speed of response, ability to work with large numbers of organisms, small fish species are being considered as supplements, or in some cases, replacements for rodent models in carcinogenesis bioassays. Simon and Lapis (1984) illustrated this potential when they used bioassays with guppies to identify carcinogenic isomers of a series of chemotherapeutics that had nitrosamine moieties. Also, in unpublished studies at our laboratory, several halogenated hydrocarbons with human health relevance were tested individually and in mixtures with medaka, guppy, and sheepshead minnow (*Cyprinodon variegatus*). In some respects, those test protocols which involved chronic exposures at stable concentrations (Walker et al., 1985) appear to be more practical than some rodent bioassays in which volatile compounds such as halogenated hydrocarbons are administered by gavage or in the drinking water.

There is a growing number of examples where small fish models can enhance our understanding of the basic mechanisms of cancer. For example, ocular cancers are terrible diseases for which there are no reliable whole animal experimental models. The medaka, however, develops retinal medulloepithelioma, a primitive type of ocular neoplasm, after a single, brief exposure to methylazoxymethanol acetate (MAM) (Hawkins et al., 1986). Similarly, MAM-induced exocrine pancreatic neoplasia in the guppy (Fournie et al., 1987) provides an additional model to study the causes and progression of that disease. Because of the sensitivity of the medaka and other small fishes to some carcinogens, the large numbers that can easily be studied, and the ability to control many extraneous factors, those species potentially provide good models for studying the biology of hepatic neoplasia and oncogene activation. Small fish models also can be used to study aspects of cancer in which large numbers of experimental organisms are required such as in low-dose risk assessment and to examine factors which only slightly increase cancer risk.

Ecological and environmental importance of cancer in fishes. Some cancer biologists and epidemiologists estimate that exposure to environmental carcinogens accounts for up to 90% of all human cancers (see Prescott and Flexer, 1986). Environmental influences on cancer prevalence are especially evident in the occurrence of cancer in wild fishes. Harshbarger and Clark (1990) document 41 geographical regions in North America in which cancer epizootics in wild fishes have occurred. They define an epizootic as a situation where three or more cases of neoplasms originating in a specific cell lineage in a geographically defined area. With regard to the types of tumors occurring, those involving hemic, neural, pigment cell, connective tissue, and gonadal neoplasms did not appear to be closely associated with environmental pollution. However, the occurrence of neoplasms of epithelial tissues such as the liver, pancreas, gastrointestinal and some epidermal neoplasms appear strongly correlated with environmental pollution.

Several cancer epizootics in wild fishes, for example English sole (*Parophrys vetulus*) from the Puget Sound, Washington winter flounder (*Pseudopleuronectes americanus*) from the Boston Harbor, and brown bullhead catfish (*Ictalurus nebulosus*) and several other freshwater species from inland waterways particularly in the Great Lakes area, have been studied in great detail and scientists are even beginning to understand some of the molecular mechanisms involved in the development of the cancers. Below, we briefly review some of those epizootics.

English sole from polluted areas of the Puget Sound have high prevalences of multiple hepatic lesions that range from degenerative lesions to neoplasms (Myers et al., 1991). Throughout numerous detailed studies (e.g., Malins et al., 1984a, 1984b, 1985, 1987, 1988) consistent statistically significant associations have been drawn between the presence of aromatic hydrocarbons in the sediments and the prevalence of the liver lesions. Further studies have focused on the metabolism of PAH's in sole liver and have included the distribution of PAH metabolites in bile and tissues and the covalent binding of genotoxic metabolites of PAH's to sole hepatic DNA (Varanasi et al., 1989a, 1989b).

The hepatic cancer epizootic and occurrence of toxic and degenerative lesions in English sole from the Puget Sound area is mirrored on the East Coast by epizootic hepatic neoplasia in winter flounder from Boston Harbor, Massachusetts (Murchelano and Wolke, 1985, 1991). As in the case of the Puget Sound sole but not as rigidly established, the hepatic lesions in the winter flounder were highly correlated with anthropogenic contamination.

Although numerous incidences of cancer epizootics have occurred in freshwater species (Black and Baumann, 1991) none have been as well studied as the English sole and winter flounder epizootics. Especially in brown bullhead catfish, PAH have generally been considered the principal causes of the neoplasia (Baumann, 1989).

Laboratory studies on the induction of neoplasia in fishes. The database on the response of small fishes to exposure to carcinogenic compounds is small but growing. In general, it appears that compounds that cause cancer in rodents also cause cancer in fishes. The variability in sensitivity to certain compounds among strains and species of rodents also occurs among fish species. Here, we review the results of laboratory carcinogenesis studies in fishes. Carcinogen response data from small fish carcinogenesis bioassays are summarized from studies and reviews of Couch and Harshbarger (1985), Hatanaka et al. (1982), and Metcalfe (1990). In the narrative below, carcinogenic responses in small fishes to representatives of several carcinogen classes including nitroso compounds, polynuclear aromatic hydrocarbons, aromatic amines, and halogenated hydrocarbons are given.

Nitroso compounds have been widely studied in small fishes with diethylnitrosamine (DEN) the most widely used compound to test for and examine mechanisms of carcinogenesis in the medaka and guppy.

It is becoming apparent that some polynuclear aromatic compounds are carcinogenic in small fishes. Some small fish carcinogenesis bioassays support the findings in wild fishes of associations between neoplasia and contamination, particularly PAH contamination. Those laboratory studies have implicated some polynuclear aromatic hydrocarbons including some well known mammalian carcinogens as causes of cancer in fishes. For example, the polynuclear aromatic hydrocarbon benzo(a)pyrene (B[a]P)is known to cause skin tumors in mammals when applied topically. The ubiquitous presence of B(a)P in sediments associated with cancer in those fishes brought to the forefront the question of whether it and associated compounds caused the hepatic and other lesions. Tests with waterborne exposures proved that B(a)P was hepatocarcinogenic to two species of small fishes, the medaka (*Oryzias latipes*) and the king cobra guppy (*Poecilia reticulata*) (Hawkins et al., 1989). Indeed, both B(a)P and a related polynuclear aromatic hydrocarbon, 7, 12-dimethylbenzanthracene appear to be far more carcinogenic in small fish models than in mammalian models (Hawkins et al., 1990).

Results of carcinogenicity studies with aromatic amines in small fishes vary. 2-Acetylaminofluroene (2-acetamidofluorene; N-2-fluorenylacetamide; 2-AAF) is an aromatic amine which as a class of chemicals, also includes the carcinogens benzidine and aniline. 2-AAF has been used as a model carcinogen to study mechanisms of initiation and promotion in rodents. For 2-AAF to be carcinogenic, it must be N-hydroxylated by a cytochrome P-450-dependent, microsomal-bound enzyme (Weisburger, 1990). Ring hydroxylation, on the other hand, by another P-450 enzyme appears to be a detoxification step. Two studies have shown that 2-AAF induces neoplastic lesions in guppies (Sato et al., 1973; Simon and Lapis, 1984). Studies described later in this report [7.0. Studies on the carcinogenicity and metabolism of the aromatic amine 2-acetylaminofluorene in the medaka (*Oryzias latipes*) and king cobra guppy (*Poecilia reticulata*)] showed some interesting results with this compound. In those studies, AAF caused a low but statistically significant increase in combined hepatic neoplastic lesions in the guppy but not in the medaka. The low carcinogenic potency of this compound, however, was evidenced by the fact that many of the induced lesions, in spite of the fact that they persisted for 6 and 9 months post exposure, did not appear robust or actively progressing to more aggressive lesions. This is the first time in our studies that we have observed the apparent regression of carcinogen-induced lesions, even ones such as those we designate as altered foci which are generally terminal lesions in rodents. The comparative carcinogenic response to 2-AAF in medaka versus the guppy, was supported by studies on the hepatic metabolism of the compound by the two species in which we showed that the guppy was more efficient in producing the carcinogenic (N-OH) metabolites than was the medaka.

Environmental and biomedical regulatory applications. Small fish systems to identify carcinogens could have extensive environmental and biomedical applications. With some environmental cancer specialists considering that a carcinogen is any chemical or compound that causes cancer of any type in any exposed organism at any dose level, the application of small fish carcinogenesis tests that are sensitive, rapid, efficient and economical could provide sound carcinogenic [illegible] on many more substances than is now presently possible to test with the expensive and lengthy rodent carcinogenesis bioassays. With regard to identifying environmental carcinogens, it can be anticipated that small fish carcinogenesis tests will soon find broad application and eventual acceptance as the definitive means of assessing the genotoxicity of aquatic sediments, effluents, and potential contaminants.

Experimental design of carcinogenesis tests with small fish species. With only a few exceptions small fish carcinogenesis tests can be designed similar to rodent tests. Factors such as numbers of test organisms, dose or concentration levels, lengths of exposure and total test length, disease surveillance, and histopathological endpoints can be arranged to parallel those in rodent tests. Fish tests can usually offer genetically consistent test organisms, uniformity of exposure to test compounds, and uniformity of test conditions such as temperature, metabolite disposal and lighting, a rapid time to tumor, a short exposure period and low cost, at least compared to rodent carcinogenicity bioassays. In this section, we will also discuss the organization of tests with respect to appropriate control groups including the use of positive control groups and experimental groups.

A few conditions that are unacceptable in rodent tests often must be accepted in most small fish carcinogenesis tests. Because many of the test species reach sexual maturity in only a few weeks or months of age, it is often necessary to hold males and females together in test systems and allow them to breed. Progeny, of course, are removed periodically from the test system. To separate the sexes possibly would place the organisms in undue endocrine stress, as many fish species are capable of changing from one sex to the other to take advantage of opportunities for dominance in social hierarchies and for establishing breeding pairs.

Another accepted condition is the unsterile environment in which the small fish are maintained. Algae and other microscopic organisms populate the grow-out aquaria, but they do not appear to affect tests and, probably, these organisms contribute favorably to the fish's nutrition and help stabilize the water quality of the aquarium.

Numerous small fish species are available as test models. Unfortunately, relatively few species have been tested for carcinogen sensitivity. Selection of test species should be based on several factors other than carcinogen sensitivity. Those include availability, economy, ease of rearing, and fecundity. Most carcinogen screening with small fishes has been conducted on the medaka (*Oryzias latipes*) and guppy (*Poecilia reticulata*), both freshwater species. Saltwater species including the sheepshead minnow (*Cyprinodon variegatus*) and the Gulf killifish (*Fundulus grandis*) offer opportunities for the development of saltwater models. Other models that have been used for specific applications are pupfish (*Poeciliopsis* spp.), zebrafish (*Brachydanio rerio*), and platyfish/swordtail hybrids.

<u>Number of animals and statistical design</u>. Statistical analysis is an important component of the interpretation of carcinogenicity tests and much effort has gone into developing sophisticated ways to analyze the tests (Gart et al., 1986). Carcinogenic activity of a test material can be reflected in an increase in the occurrence of neoplastic lesions in the test organism with the increase being in the rate of occurrence of an otherwise spontaneously occurring lesion or in the occurrence of a lesion that does not typically occur spontaneously. The latter situation is probably accepted by most as the more valid indicator of carcinogenesis. Another effective measure of a carcinogen is a decrease in the time-to-tumor. Most agree that a chemical carcinogen should cause a statistically significant increase in a specific type of tumor in treated versus non-treated groups. Guidelines have been established, however, that for statistical analyses allow pooling certain types of lesions based on their cells of origin. Furthermore, it is expected that the incidence of the tumor increases with increasing dose of carcinogen (Peck, 1973).

The low rate of spontaneous neoplasia in fish species is a distinct advantage that these models have compared with rodent models. In practical terms, the lower the spontaneous rate of neoplasia, the fewer the number of specimens needed to achieve statistical significance of the results. For example, Krewski et al. (1989) calculated that to statistically validate a 10% tumor incidence when the spontaneous rate is 1%, 161 test animals, half assigned to the test group and half assigned to the control group, are needed. The number of test animals needed more than doubles however, if the spontaneous tumor rate increases to 10% or a statistically valid tumor rate of 5% over the spontaneous rate is sought.

<u>Determination of concentration of test compounds, length of exposures, and lengths of tests</u>. In this section, we discuss and suggest guidelines for determining the concentrations of test compounds in the experimental groups, length of exposures, and schedules for sampling.

Dose or concentrations can vary with study objectives. The low cost of small fish carcinogen bioassays compared with rodent tests affords a great deal of flexibility in organizing tests with regard to numbers of exposure groups and sampling schedules. Dose selection is an important issue in rodent carcinogenicity tests. Pointing to the difficulties of carcinogenicity tests to identify weak and moderately strong carcinogens and the limitations of using necessarily small sample sizes because of economic restraints, Haseman (1985) suggested that exposure concentrations be based on a "maximum tolerated dose" (MTD). We agree that for carcinogen testing with small fish efforts should be made to avoid false negative results. To avoid false negatives, the high exposure group should be near the toxic limits for the exposure period. One approach might be to have three exposure groups with a high level that is an established percentage (e.g., 25%) of the lethally toxic concentration, a low exposure concentration at or near real environmental levels, and an intermediate concentration somewhere between those two. The idea behind having three or more exposure groups is not necessarily to establish a dose-response curve but to help insure against a false-negative outcome. Appropriate control groups would include an untreated group, a shame-treated one in case the experimental conditions differed from those of grow-out, and one that might be treated with a solvent or other type carrier used for the test compound.

Under some circumstances, a positive control group might be included in a test. A positive control group in a carcinogenicity test is one in which the experimental organisms are exposed to a compound, for example a proven carcinogen in the test model, that will induce neoplastic lesions. The use of a positive control treatment group has both positive and negative attributes. On the negative side, carcinogens operate by such specific mechanisms that a response to a given compound might only mean that the organism is sensitive to that compound alone (Peck, 1973). On the other hand, standardization of the use of positive controls could help establish the potency of the test compound by comparing the response it induces with that of a proven carcinogen (Weisburger, 1973) and with the results of prior tests. Furthermore, a positive control group can indicate if conditions differ from those of prior tests.

Typically, the duration of small fish tests is no more than one year. Although this does not represent a "life span" test it is probably a practical length in that it appears that if a carcinogenic response is to be observed it occurs within one year. Furthermore, longer studies run the risk of disease outbreaks, failure of support systems, and development of spontaneous neoplasms. Under some circumstances, however, studies longer than a year can be instructive such as in the detection of very low carcinogenic potencies and allowing for the development of extrahepatic neoplasms which typically develop slower than hepatic neoplasms. In other cases, tests may be terminated at a scheduled time of 4 to 6 months or earlier than the projected duration. Consideration should be given to preserving adequate numbers of test organisms so that sufficient statistical and pathological information can be collected. In the case of tests that have not run for more that six to nine months, consideration should be given to re-starting the tests.

Determination of histopathological sampling schedules depends on several factors including the expected carcinogenicity of the test compound and its target organ. It is often useful to examine a small number of specimens for acute toxicological effects to estimate toxic exposures and, in some cases, predict onset of carcinogenesis. All moribund and in some cases dead specimens should be examined histologically to determine, if possible, the cause of death. In interim samples, all high concentration specimens should be examined histologically. When positive responses are observed, intermediate and low concentration exposures and controls should be examined. A strength of the small fish carcinogenicity test is that it easily affords the opportunity to examine whole specimens for carcinogenic effect usually on one or a few slides.

Exposure systems and chemistry support. Small fish carcinogenesis tests are highly versatile offering a wide range of approaches to testing suspect cancer-causing compounds. We will describe and discuss in detail three principal systems including flow-through exposures, static-renewal exposures, and static exposures. Other exposure methods including dietary exposures, embryo injection, intraperitoneal injection will be discussed. We will also emphasize in this section the necessity of accurate environmental chemistry backup data for the carcinogenicity tests.

Decisions on the exposure methodology to be used are based on a number of factors such as the availability of the test compound, its toxicity to test organisms, expected carcinogenicity, solubility, ease of disposal and possibly other factors.

Evaluation of toxic responses. Carcinogenic compounds often produce in target organisms toxic responses that may or may not be related to carcinogenic responses that develop subsequently. Here, we will examine approaches to evaluating the toxicity of test compounds especially in relation to carcinogenicity determinations. Effects on mortality, growth, and fecundity will be discussed along with second generation effects such as teratogenesis.

Evaluation of carcinogenic responses. The induction of neoplastic lesions is typically the primary endpoint of a carcinogenesis test. In this section, we will describe and discuss methods for assessing the pathological indicators that confirm that carcinogenic events have taken place. Histological methods will be detailed including sampling procedures, fixation, embedding, sectioning, and staining. Criteria for the histopathological evaluation of neoplastic lesions will be discussed. New methodology including the use of immunocytochemical techniques to specifically characterize neoplastic lesions and morphometrical techniques to quantitate neoplasm number and size, progression and carcinogen potency will also be discussed.

Personnel safety and disposal of test materials. Worker safety and the proper disposal of hazardous materials generated in the laboratory are as important in carcinogenesis tests as in any other laboratory situation. Any laboratory exposure to a carcinogenic hazard is considered unacceptable and appropriate guidelines for storing, handling, and disposing of carcinogens must always be followed.

Interpretation of results. Carcinogenic mechanisms as they are presently understood for the primary small fish models will be discussed briefly primarily to assure the reader and potential user that mechanisms of carcinogen activation and detoxification and oncogenetic processes are no more or less predictable or rational than in rodent systems. Differences in fish systems with respect to mammalian systems, for example, the lack of an active phenobarbital-metabolizing MFO system will be discussed.

Inherent in assessing the carcinogenic effects of a test substance is an understanding of the spontaneous neoplastic lesions known to occur in the experimental models. As mentioned earlier, the low frequency of spontaneous neoplasms in small fishes is an important attribute. Data indicate that in the small fish models, the primary target of most carcinogens is the liver, particularly hepatocytes and biliary epithelial cells. Both the medaka and guppy from our cultures develop spontaneous hepatic neoplastic lesions. Incidences of hepatocytic neoplastic lesions are low for the 24, 36, and 52 week periods that are typically sampled in carcinogen screening assays. Incidences for cholangiocellular lesions are even lower.

Information is scanty, however, on the etiology and toxicologic pathology of non-hepatic neoplastic lesions and not enough data are available for us to distinguish between non-hepatic spontaneous lesions and carcinogen-induced ones. Some lesions are clearly inducible by carcinogens such as retinal medulloepithelioma in medaka by methylazoxymethanol acetate (Hawkins et al., 1986) and exocrine pancreatic neoplasia in the guppy by that same compound (Fournie et al., 1987). Several other non-hepatic lesions appear to be spontaneous and not carcinogen inducible, at least not with the carcinogens we have examined. For the medaka, these include pancreatic acinar carcinoma (Hawkins et al., 1991), thymic lymphoblastoma (Battalora et al., 1990) and germ cell neoplasms that resemble spermatocytic seminoma (Hawkins et al., Submitted). For the guppy, retinal adenocarcinoma appears to be spontaneous (Fournie et al., 1992). It also appears that neoplasms of the swim bladder epithelium that have appeared in medaka and guppies from our laboratory are spontaneous and not carcinogen induced.

Masahito et al. (1989) provided data on life-span tumor development in medaka. They examined nearly 1000 medaka from one to five years of age. Liver tumors were rare in one-year old specimens but the incidence rose to 7.1% in five-year-old females. Spontaneous tumors were rare in other organs and included squamous cell carcinomas, melanomas and lymphosarcomas none of which occurred in relation to sex or age.

We recommend that if the occurrence of a rare neoplasm in the small fish species is considered to result from carcinogen exposure, the decision should be made cautiously and that both historical and concurrent cases of neoplasia in controls should be used to evaluate a carcinogenic response. The occurrence of such lesions could be highly relevant in assessing the carcinogenicity of a test substance even in the absence of statistical significance. On the other hand without an extensive database of spontaneous lesions occurring in control specimens, this situation could result in a false positive error.

Accurate assessment of carcinogen-induced lesions is critical to the properly conducted carcinogenesis test. Although it is becoming scientifically sophisticated, the histopathological evaluation of carcinogen-induced lesions in fishes does not require nearly the level of skill or professional training that the assessment of toxic and carcinogenic lesions in mammals requires. The liver is the principal target organ although recent small fish studies from several laboratories are showing that carcinogenic potency can be estimated by the rate of occurrence of extrahepatic primary neoplasms that develop subsequent to the occurrence of hepatic neoplasia. The principal extrahepatic neoplasms in small fishes is highly species specific, for example, ocular neoplasia in medaka and exocrine neoplasia in the guppy. Other carcinogen-induced nonhepatic neoplasms in small fishes include renal, gill, vascular, and neural neoplasms.

In rodent tests, it is advisable to distinguish between benign and malignant lesions (D'Aguanno, 1973). Malignant lesions are considered those that ultimately kill the organism. Most of the more common carcinogen-induced lesions in small fish do not kill over the test periods of a year or so. Histologically, however, many of the lesions have the hallmarks of malignancy or demonstrate the capability of progressing to malignancy. We recommend that at this stage of development of the small fish carcinogenesis bioassay carcinogen-induced lesions are not distinguished according to whether they are benign or malignant until considerably more information concerning the biology of neoplasia in small fish is known.

Confidence in the results of small fish carcinogenesis bioassays will increase as concordance between the results of small fish tests and rodent tests occurs. It is also important that the test results be interpretable in the light of what is known of the metabolic mechanisms by which test models handle carcinogens. A further objective should be the incorporation of ancillary in vivo tests to the fish bioassays. The have been discussed by Weisburger and Williams (1991) and include a battery consisting of the Ames test (a reverse mutation assay in prokaryotic *Salmonella typhimurium*), assay for DNA repair in explanted liver cells, determination of DNA adducts, DNA breakage, chromosome aberrations, sister chromatid exchange.

Numerous pitfalls are associated with the histopathological evaluation of a carcinogenic response. There appear to be several toxic lesions associated with carcinogenic responses and, furthermore, target organ toxicity may be a critical stage in the process of the development of neoplastic lesions. Additional lesions seem to reflect non-carcinogenic toxicity or, at least, toxicity that is not part of the neoplastic process. Some inflammatory lesions in fishes also mimic neoplastic lesions. These should be considered from the standpoint of distinguishing neoplastic from non-neoplastic proliferative lesions.

Statistical analyses. Properly designed, small fish carcinogenesis tests offer exceptional opportunities to statistically analyze study results. In addition to tumor incidence, other components that can be analyzed include tumor-associated mortality, patterns of tumor co-occurrence, tumor lethality and regression and the comparison of malignant versus benign tumors, time of tumor onset and dose response.

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3.0. Methodology

3.1. Fish Culture and Maintenance Techniques

Medaka *(Oryzias latipes)*. Brood stock are maintained at a 3:2 female:male ratio with a density of about 5 fish per gallon in 10-gallon or larger aquaria containing well water that has been aged for a minimum of 24 hours. Brood tanks are held at 25-28°C and provided a daily light regime of 16 hours light and 8 hours dark. Fish are fed dry food (Prime Flakes, Zeigler Bros., Inc.) sparingly 3 times daily and are provided live brine shrimp *Artemia* nauplii once daily. Feces and other debris are removed from the aquaria twice each week, with a concomitant water change of at least 20%. Aeration and continual water filtration are provided by biological sponge filters.

Eggs can be collected by siphoning them from the tank bottom, by teasing them away form a netted female, or by removing them from biological sponge filters following egg deposition by the female. Individual eggs are placed in hatching solution in one gallon aerated jars at a density not exceeding 1 egg per milliter. Temperature is maintained at 24°C. Hatching solution contains 100 mg NaCl, 3 mg Kcl, 4 mg $CaCl_2.2H_2O$, and 16.3 mg $MgSO_4.7H_2O$ per 100 ml glass distilled water. Dead or diseased embryos are discarded. As fry begin to hatch (about 10 days following fertilization), they are transferred to finger bowls containing approximately 1400 ml water with an average of forty fry per bowl and provided ciliates for 3 days and nematodes for 6 or more days. Bowls are maintained in a 27°C water bath. Feces and debris are removed daily with a concomitant 50% water change to insure good water quality. After 3 days, newly hatched *Artemia* nauplii are added as a food source, and after 6 to 10 days fry are fed dry food and *Artemia*. Fry are either utilized for testing at or about age 6 day or transferred to maintenance aquaria to provide future brood stock. Water quality, temperature, and manipulations in maintenance aquaria are as described for brood aquaria with the exception of a 12 hr:12 hr light:dark cycle.

Guppy *(Poecilia reticulata)*. Initial guppy brood stock were purchased from Aqua World, Inc., St. Louis, Missouri. Brood guppies are maintained in aquaria ranging from 20 to 30 gallons at a loading density of approximately 2 fish per gallon and a 3:5 male:female ratio. Aquaria are filled with well water aged a minimum of 24 hours and are equipped with biological sponge filter systems. Temperature is maintained at or near 27°C, and pH ranges from 8.0 to 9.3. Feces and debris are removed twice weekly, and 20% of the water is changed. Newborn fry are afforded refuge in a thick floating vegetative mat of hornwort. If cover is limited, gravid females are often consolidated in 20-30 gallon aquaria for short periods of time to facilitate fry collection, following which females are returned to tanks with males. Brood rooms are exposed to natural sunlight and enhanced by artificial photoperiod of 12 hours light and 12 hours dark. During periods of fry collection, however, photoperiod is increased to 16:8 light:dark. Brood fish are fed dry food (Stress Flakes, Aquavet) three times daily and live artemia nauplii once daily.

Several weeks after birth, the male guppy develops a "gonopodium" which is a rod-like structure on the fish's underside that runs parallel to the body. At the time of fertilization, the gonopodium of the male enters the vent of the female delivering sperm which fertilizes the eggs. Shortly thereafter, the female develops a "gravid spot" in the shape of a triangle near the vent. The spot becomes darker and gradually enlarges giving the female a swollen appearance. When viewed from above or laterally, the gravid spot becomes more pronounced as the incubation period progresses until the time young are born. Guppies deliver as few as two or three fry, or as many as one hundred in one brood, and may deliver their young at various periods of time following the same fertilization. In collection aquaria, feeding and grassed brood areas are divided by 6-inch plexiglas strips to prevent food from entering protective vegetated areas. Fry are collected by netting and are maintained at a density of 50-80 fry in 5-gallon aquaria filled with water from the brood aquaria. Fry aquaria are maintained under feeding, lighting, filtration, and temperature regimes identical to those in brood aquaria.

3.2. Exposure Techniques

Static, Single-Pulse Exposures. Static, single-pulse exposures with medaka are conducted in a commercial carcinogen glove box (Labconco 50350) in aged non-chlorinated well water. Exposures are routinely conducted in the dark at a water bath- maintained temperature of 26 $\pm$ 0.5°C. Duration of exposure varies among chemicals from 2 to 24 hours. Test fish that are 6-10 days old are selected on the basis of uniformity of size and health from the total lot available and are housed in Nytex mesh chambers within glass beakers or small aquaria during the exposure. Fish are not fed during exposure. Following exposure, fish are rinsed with toxicant-free water and transferred to 38-liter grow-out aquaria maintained at 27°C under a 12 hr:12 hr light:dark regime. Fish in grow-out are fed dry food three times and *Artemia* nauplii once daily. Exposed fish are observed at least once daily for indications of stress or aberrant behavior and are sampled at predetermined intervals and histologically processed for light and on occasion electron microscopic examination.

Intermittent Multiple-Pulse Exposures. Intermittent, multiple-pulse exposures are conducted under conditions described above for the static, single pulse exposures. Exposures are performed at weekly intervals through a maximum of 6 weeks. Between exposures, fish are maintained within their meshed chambers in toxicant-free water at 26 $\pm$ 1°C under a 12 hr:12 hr light:dark regime and fed as described above. Dead, dying, and diseased fish are removed upon observation. Upon conclusion of the final exposure in the series, fish are rinsed and transferred to grow-out aquaria.

Flow-Through Exposures. Flow-through exposures of variable duration are conducted in a specially designed flow-through glove box currently in use in our laboratory and described by Walker et al. (1985). Toxicant-free control treatments accompany all exposures. To maintain consistent concentrations of test chemicals throughout an extended exposure period, a toxicant reservoir consisting of 2 or 3 serially connected sealed 45.4 L pyrex carboys is utilized. Test chemicals and test water are added to each carboy, and the contents magnetically stirred until the concentration of test chemical stabilizes at or near its saturation limit.

When a test is initiated, toxicant-laden water is withdrawn from the nearest, or dispensing, carboy in the series by precision liquid dispensing syringe pumps (PLD-II, Hamilton Company, Reno, NV) and delivered through microbore tubing to a maximum of six mixing chambers. Toxicant-free water enters the system by gravity flow from an elevated head box through a solenoid-controlled valve, filling a 7-compartment water partitioner similar to that described by Schimmel et al. (1974). Float switches within the water partitioner activate a programmable laboratory controller (Idec PLE-30R, Industrial Electric Supply Co., Birmingham, AL) which in turn activates the series of PLD injectors. All injectors draw from the dispensing carboy but receive different instructions from the controller regarding number of injections per cycle. The flow of diluent water into the water partitioner is controllable by design to provide a range of cycling times. Syringe-size, distance of plunger withdrawal, and number of injections can be varied, thereby facilitating introduction of a wide variety of toxicant masses and hence test concentrations. For chronic exposures (3-6 months), 48-liter aquaria are usually used, with 250-300 fish per aquarium. In this configuration, the exposure system can handle three chemical concentrations and a control. Fish can be contained in meshed chambers (10 cm ID petri dishes, each with a 9 cm high nylon mesh collar) or allowed to swim freely within treatment aquaria. Each treatment aquarium fills to a depth of 8 cm, at which time toxicant-laden water discharges through activated carbon (Filtersorb 400, Calgon Corp., Houston, TX). Mixing chambers, splitter boxes, and treatment aquaria, all constructed of glass and silicone cement, are housed within a 341.6 cm long by 92.7 cm wide by 53.3 cm high resin-coated plywood exposure chamber covered with a pitched top, 34.3 cm high along its center. Ingress and egress is accomplished through capped ports, and manipulation of materials within the chamber is conducted through 3 gloved ports along each side of the chamber. Treatment aquaria are housed within a central water bath maintained at 26 ± 1°C in a 12 hr:12 hr light:dark regime. The exposure chamber is maintained at a slight negative pressure by exhaust fans which also serve to draw incoming air and remove gaseous toxicants through carbon filters (BPL activated carbon, 12 x 30 mesh, Calgon Corp., Houston, TX). Generally, fish are alternatively fed Stress Flakes and *Artemia* nauplii throughout the exposure period. Fish are observed periodically each day during exposure, and dead fish are removed and recorded upon discovery. Toxicant concentrations are monitored once or twice each week throughout each exposure period by appropriate gas-liquid chromatographic methods. Upon termination of exposure, fish are transferred to grow-out aquaria and maintained and sampled as previously described.

3.3. Grow-Out Techniques

Following exposure, fish are placed in 38-liter aquaria (maximum 100 fish/aquarium) containing aged well water. Aquaria are held in circulating water baths at 26 ± 1°C under a 12 hr:12 hr light:dark regime. Aeration and continuous water filtration are provided by biological sponge filters. Feces and debris in the tanks are removed, effecting a 20% water change, 3 times weekly. Temperature, pH, salinity, and ammonia are monitored monthly on a revolving tank schedule to insure optimum water quality. Fish are fed dry food 3 times daily, and live *Artemia* nauplii once daily.

3.4. Histological Procedures

Our histological procedures are generally designed to allow us to examine large numbers of specimens and to survey most internal organs of each individual specimen. Usually, whole adult fish specimens are processed. Fish are narcotized in ice water or MS-222 (tricaine methanesulfonate), the bellies of larger specimens are slit open and whole fish are placed into Lillie's fixative (10% formalin; 85% saturated aqueous solution of picric acid; 5% formic acid). This solution decalcifies as well as fixes. Depending on the size of the specimen, fixation lasts 24 hours to 1 week. Extended fixation times insure decalcification of the larger specimens. Specimens are then dehydrated in ethanol, cleared in Americlear, and embedded in paraffin. Several small fish specimens can be embedded in a single paraffin block. Specimens about six-months old and older are mounted individually. Usually, fish are embedded on one side or the other depending on which side the liver lies. To survey most major organs, sections are taken from a parasagittal plane, mounted on glass slides, and routinely stained with hematoxylin and eosin. In some cases, specific tumors are sectioned selectively. For example, eye tumors are probably best studies in transverse sections, whereas neurogenic tumors, most of which arise from spinal nerves, require sagittal sections through the spinal column to visualize early stages and origins of those tumors. When appropriate, special stains are used to identify cellular components, secretory products, or tissues. Slides are read and diagnoses recorded without the histopathologist knowing the specimen's exposure history. Non-neoplastic lesions are described. Each tumor is described and measured.

3.5. Electron microscopy

For electron microscopical studies, tissues are dissected, immersed in 3.0% glutaraldehyde in 0.1 M phosphate buffer, rinsed 2 hours to overnight in buffer, and postfixed in 1.0% osmium tetraoxide in phosphate buffer. Tissues are dehydrated in ethanol, soaked in propylene oxide, and embedded in one of several commercially available epoxy resins. To take advantage of the improved resolution of plastic-embedded tissues and to choose areas for thin sectioning, 2 μm thick sections are cut, stained with toluidine blue, and examined with a light microscope. Thin sections are cut on an ultramicrotome, mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with an electron microscope.

3.6. Randomization and sampling

There are junctures in aquatic toxicology tests in which randomization is critical to insure validity and reproducibility of the data. Whereas it is not practical from the standpoint of time and resource expenditure to attempt to control all extraneous factors in toxicity tests, we believe two situations to be particularly important: 1) introduction of cultured specimens into the test environment, and 2) selecting specimens from test systems for examination, either from exposure or growout. Our methods for addressing those factors are described below. Other factors that we consider to be less important in that their effects might be seen only in tests with high statistical precision such as when large numbers of specimens are used, include randomization of the placement of aquaria in exposure and growout systems, randomization of specimen processing, and fully "blinded" slide reading.

Methods for randomization of specimens for exposure are as follows. Test fish of the appropriate age are counted and distributed according to a scheme involving computer-generated random number sequences. Initially, a set of random numbers equal to the number of treatments (control plus exposure groups) is generated. The number of these sets will depend on the desired total number of specimens in each treatment and the number of specimens to be added for each set. For example, if there are 300 fish per treatment and 10 fish are to be added in each pass, 30 sets (300 divided by 10) would be needed. These 30 passes would be divided among the technicians counting the fish specimens. For example, if there are 5 treatment groups in the test and two technicians are counting fish, each technician would have 2 sets of 5 beakers. They would draw fish from a common reservoir and pour 10 fish into each of the 5 beakers filling the #1 beaker first and the #5 beaker last. Once the beakers each contain 10 specimens, the fish are poured into the corresponding treatment beaker (the container in which the specimens will be exposed). There is one treatment beaker for each treatment group and they are numbered. For example, the random number set may dictate that the contents of fish beaker #3 be added to treatment beaker #1, and so on. To estimate specimen weight at the beginning of a test, a separate beaker of fish is incorporated into the scheme to receive 50 specimens that are subsequently weighed. This procedure assures that weight variability within the population of test fish is also randomly distributed throughout the treatments.

The following is an example illustrating how this system works:

Suppose the random number set for 5 treatments was

4	3	1	5	2

The left to right order of these numbers corresponds to the #1 though #5 fish beakers. The technician pours the fish from his fish beaker #1 into treatment beaker #4, and from his fish beaker #2 into treatment beaker #3, and so on. There will be as many of these passes as are necessary to get the final desired number of fish in a treatment beaker. Because there are two technicians, each will make 15 such passes. Then, numbers are randomly assigned to combine the treatment beakers of each technician. For example,

3	1	4	2	5
2	1	5	3	4

Therefore, treatment beaker #3 of technician #1 will be combined with treatment beaker #2 of technician #2 and so on.

The final set of random numbers determines which exposure group a final treatment beaker will become.

4 3 1 5 2

In this case, the lowest exposure concentration (such as control) will receive the final treatment beaker #4 and so on to the highest concentration receiving final treatment beaker #2. The left to right order corresponds to increasing treatment concentration.

Methods for sampling specimens from exposure or grow-out containers are much less involved than those described above but are also designed for randomization. For example, in a typical test, samples of 100 specimens from each treatment group (control and exposure groups with each group maintained in four replicate aquaria) would be taken for histopathological examination at 6, 9, and 12 months following the initiation of exposure. Specimens are removed from grow-out aquaria by netting. Fortunately, medaka and guppies are rather slow-moving fishes which makes it unlikely that weaker specimens are selectively netted. To sample 100 specimens, fish are randomly netted in five rounds, with five fish removed per round from each of the four replicate aquaria that comprise each treatment and control group. Specimens are identified according to grow-out aquarium, and provided in segregated fashion for histopathological analysis. Replicate identity is maintained throughout processing and evaluation.

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4.0. Absence of carcinogenic effects in guppy and medaka following exposure to the halogenated hydrocarbons chlorodibromomethane and tetrachloroethane

Introduction

1,1,2,2-Tetrachloroethane (TeCE) is a solvent used in cleaning processes and in the manufacture of paints, varnishes and rust removers. Chlorodibromomethane (CDBM) occurs in municipal drinking waters as a result of the interaction of organic materials with byproducts of chlorination. TeCE and CDBM were shown to be carcinogenic when administered by gavage to B6C3F1 mice but were not carcinogenic when administered to F344 rats. The compounds cause hepatic adenomas and carcinomas in female mice and adenomas only in male mice (Haseman et al., 1984). Because TeCE and CDBM are contaminants of ground supplies of drinking water, they are considered potential health threats and are on EPA's Priority List of Drinking Water Contaminants (EPA, 1988).

We conducted carcinogenesis bioassays with TeCE and CDBM against the medaka and the guppy in flow-through exposures. Histopathological examination of specimens exposed to TeCE or to CDBM for three months then grown-out in clean water for additional periods of three, six or nine months did not indicate that these compounds were carcinogenic to the fishes.

Experimental methods and results

Details of methods and procedures for small fish culture, range-finding tests, the exposure apparatus, exposure protocols, analytical chemistry, grow-out, and histological analyses are described above in section *3.0. Methodology.*

For the medaka tests, three hundred 6-day-old fry (mean wet and dry weights of 3.34 and 0.53 mg/fry, respectively, based on a random sample of 50 similarly aged fry) were utilized. For the guppy tests, 300 fry (wet and dry weights of groups used for individual treatments ranged from 8.87-9.0 mg/fish and 1.67-2.46 mg/fish, respectively) with exception of the aquarium control group which received only 260 guppies because of the low production for that particular exposure. Guppies were less than or equal to 48-hours postpartuition when introduced into the treatment groups.

Test specimens were randomized as described above in section *3.6. Randomization and sampling* and assigned to the following treatment groups.

For the TeCE test, the experimental groups were as follows:

(1) Aquarium control group (situated outside the exposure system)

(2) Flow-through control group (situated inside the exposure system)

(3) Low exposure group (low concentration of TeCE administered continuously for 90 days)

(4) Intermediate exposure group (mid-concentration of TeCE administered once weekly for 24 hours throughout the 90 day exposure period)

(5) High exposure group (high concentration of TeCE administered once weekly for 24 hours throughout the 90 day exposure period)

For the CDBM test, the experimental groups were as follows:

(1) Aquarium control group (situated outside the exposure system)

(2) Flow-through control group (situated inside the exposure system)

(3) Continuous exposure group (continuous CDBM exposure for 90 days)

(4) X 1 intermittent exposure group (CDBM administered once weekly for 24 hours throughout the 90 day exposure period)

(5) X 2 intermittent exposure group (intermittent CDBM exposure administered twice weekly for 24 hours throughout the 90 day exposure period)

About 100 specimens from each treatment group were sampled for histopathological examination at 24, 36, and 52 weeks post-initial exposure.

Exposure solutions were delivered to the test aquaria by means of a water-partitioning dilutor system. Three Hamilton PLD-II syringe pumps, each equipped with either a 5-ml or a 10-ml glass syringe, withdrew CDBM or TeCE stock solution from the nearest of three tiered and serially-connected 45L glass carboys, each mixing atop a magnetic stir plate. Stock solution was prepared by adding 150 g of CDBM or TeCE (Aldrich Chemical Company; 98% purity) to 45 L of well water, and then mixing the solution for several days. The top carboy in the tiered series was replaced on exposure days 9 and 36 as stock solution was depleted. On exposure day 60, the top carboy was permanently removed from the series to minimize wastage and disposal of toxicant stock solution at the end of the study.

A non-chlorinated artesian well located on the GCRL campus was the source of diluent water. Well water entering the building was stored in a 250-gallon fiberglass reservoir, where it was heated and recirculated, before being pumped through a 20 μm canister filter to the headbox which fed the dilutor system's chambered water partitioner. Twice weekly, samples were removed from the 250-gallon reservoir to monitor the dissolved oxygen and pH of the dilution water. Dilution water temperature was checked on a more frequent basis.

Stock solution and diluent water were blended in each of 5 mixing chambers positioned above a pair of test aquaria. Each mixing chamber emptied into a splitter box, which dispensed approximately one liter of test solution per cycle to each of the two test aquaria below it. One of the two test aquaria contained medaka and the other contained guppies. Test aquaria were 24.5 X 11 X 9-inches, made of glass, and provided with an overflow drain which maintained test solution volume at approximately 30 liters. The toxicant delivery system was set by means of a binary timer to cycle a minimum of 6.25 times per hour, a rate which provided at least 5 volume additions per day.

Light was provided by fluorescent bulbs mounted above the test chambers. Photoperiod was 16 hours light, 8 hours dark. Test aquaria were located in a water bath, which was heated to maintain the temperature of the test solution at 27 $\pm$ 1°C.

The definitive studies were begun by assigning 300, 6-day post-hatch medaka or 300, 24 to 72 hour-old guppies into each of the control and treatment aquaria. An additional 150 specimens of each species were also placed in groups of 30 into retention chambers, one of which was added to each of the control and treatment aquaria. For each study, two control aquaria, each receiving diluent water only, were included for each species of fish. One, the flow-through control, was located within the exposure chamber with the CDBM- or TeCE-treated aquaria. The other, the aquarium control, was located outside of the exposure chamber to preclude CDBM or TeCE contamination due to volatilization and aspiration.

Measured TeCE concentrations in the medaka and guppy studies are shown in figures 4.1 and 4.2, respectively. Mean TeCE concentrations for the low (continuous) exposure were about 3.97 mg/L in the medaka test and 3.45 mg/L in the guppy test. A concentration spike up to about 10 mg/L occurred at one period in low concentration in both tests and was immediately corrected. The spike was caused by an inadvertently disconnected airline to the pneumatically operated toxicant injector. The mean intermediate concentration (administered once a week for 24 hrs) was about 7.76 mg/L in the medaka test and 6.93 mg/L in the guppy test. The high concentration (also administered once a week for 24 hrs) was about 13.93 mg/L in the medaka test and 12.78 mg/L in the guppy test.

In the CDBM test, fish were exposed to a single nominal 6.0 mg/L concentration of the compound in each of the three exposure groups. In one group, the fish were continuously exposed to CDBM at a mean concentration of about 5.89 mg/L in the medaka test (Figure 4.3) and 5.34 mg/L in the guppy test (Figure 4.4). In the other two treatments, the same nominal concentrations were delivered to the aquaria on an intermittent basis by turning the appropriate syringe pumps on or off. In one intermittent treatment (1X), toxicant was delivered to the test aquaria for 24 hours once a week. That mean concentration for the medaka test was about 6.50 mg/L and 5.39 mg/L for the guppy test. During the remaining six days out of each week, diluent water only flowed into these aquaria. In the second intermittent treatment (X2), toxicant was delivered to the test aquaria for two 24-hour periods each week. That mean concentration was 5.78 mg/L for the medaka test and 5.28 mg/L for the guppy test. Toxicant delivery periods were separated by two, then three days, of diluent water only flowing to these tanks.

Throughout the CDBM study, medaka in the continuous treatment were observed to be relatively inactive compared to control fish. Particularly during the early part of the study, the continuously-exposed medaka exhibited jerky swimming movements. Guppies from the same treatment were also observed to be quiescent compared to controls. Mortality was monitored daily by visual inspection of each test chamber and by examination of debris siphoned from the tanks at least once a week. Significant mortality was not noted. Temperature, pH, and dissolved oxygen in exposure aquaria were measured twice weekly throughout the test. Water samples for the gas chromatographic determination of CDBM or TeCE concentrations were collected twice weekly from each control and treatment aquarium, 20-22 hours following the initiation of toxicant delivery to one or both of the intermittent treatments. At two or three week intervals, four additional water samples were removed from the 1X and 2X aquaria a few hours prior to turning on toxicant delivery to both intermittent treatments. The purpose of these samples was to verify that the fish were residing in relatively CDBM- or TeCE-free water between pulses. These off-day measurements ranged from 0 to 0.008 ppm in the 1X aquaria and from 0.008 to 0.017 ppm in the 2X aquaria.

On exposure day 10 of each study, specimens in the retention chambers were removed briefly from the exposure aquaria to be photographed with a Polaroid MP-3 camera. Polaroid prints are archived to be evaluated for differences in lengths of fish from the various treatments. On day 30 fish in the retention chambers were removed and counted. Fish from each retention chamber were examined for possible compound-related growth reduction. Each of these fish was measured to the nearest millimeter for standard body length, and individually wet and dry weighed. During the processing of fish from the retention chambers, the continuously-exposed medaka were noted to exhibit marked abdominal distension.

A one-way analysis of variance, followed by a two-tailed Dunnett's test which compared each treatment to the flow-through control, was applied to the growth data for each fish species. Results indicate that intermittent exposure to 6ppm CDBM was not detrimental to the growth of young medaka. However, continuous exposure to 6ppm CDBM for 30 days resulted in statistically significant reductions in standard length and wet weights.

On the other hand, the growth of young guppies was not significantly retarded relative to the controls in either the continuous or intermittent CDBM treatment aquaria.

On exposure day 77, daily egg collections were begun in the medaka aquaria and continued for two weeks through day 90. Eggs were collected by removal from six sponge filters added to each medaka tank on day 76. Eggs were counted and examined to determine viability. Viable embryos were placed into petri dishes containing a hatching solution. Embryos were kept segregated according to both treatment and day of collection. Embryonic development was followed daily for 10 days post-collection. Abnormalities and dead embryos were tallied. Preliminary observations suggest that continuously-exposed medaka were the only treatment group to produce no eggs during the collection period.

Following 90 days exposure, toxicant delivery to the exposure aquaria was terminated. Fish were allowed to recover for 9 days before their removal from the test chambers on day 99. A random sample of 100 fish of each species from each treatment was photographed for future examination to ascertain growth effects or abnormalities. Fish were then transferred to grow-out aquaria for future histopathological studies.

With both species, neither exposure nor grow-out mortality were dose-related and more that 92% of each species from each treatment group survived to grow-out. Histological examination of three whole specimens of each species from each treatment group taken at the end of the 90-day exposure did not reveal any toxicant-related pathological effects.

The results of histopathological examination of medaka and guppy exposed to TeCE are summarized in Tables 4.1 and 4.2, respectively, with incidences of combined hepatic neoplastic lesions given. Because significant incidences of neoplasms were not seen in the high exposure group, only one control group or group exposed to lower TeCE concentrations was examined. Based on this analysis, TeCE did not appear to be carcinogenic to either the medaka or the guppy.

Similarly, as shown in Tables 4.3 and 4.4, CDBM did not appear to be carcinogenic to either the medaka or the guppy, respectively. At this point, we have not determined the histopathological cause of the abdominal distention seen in specimens from interim exposures.

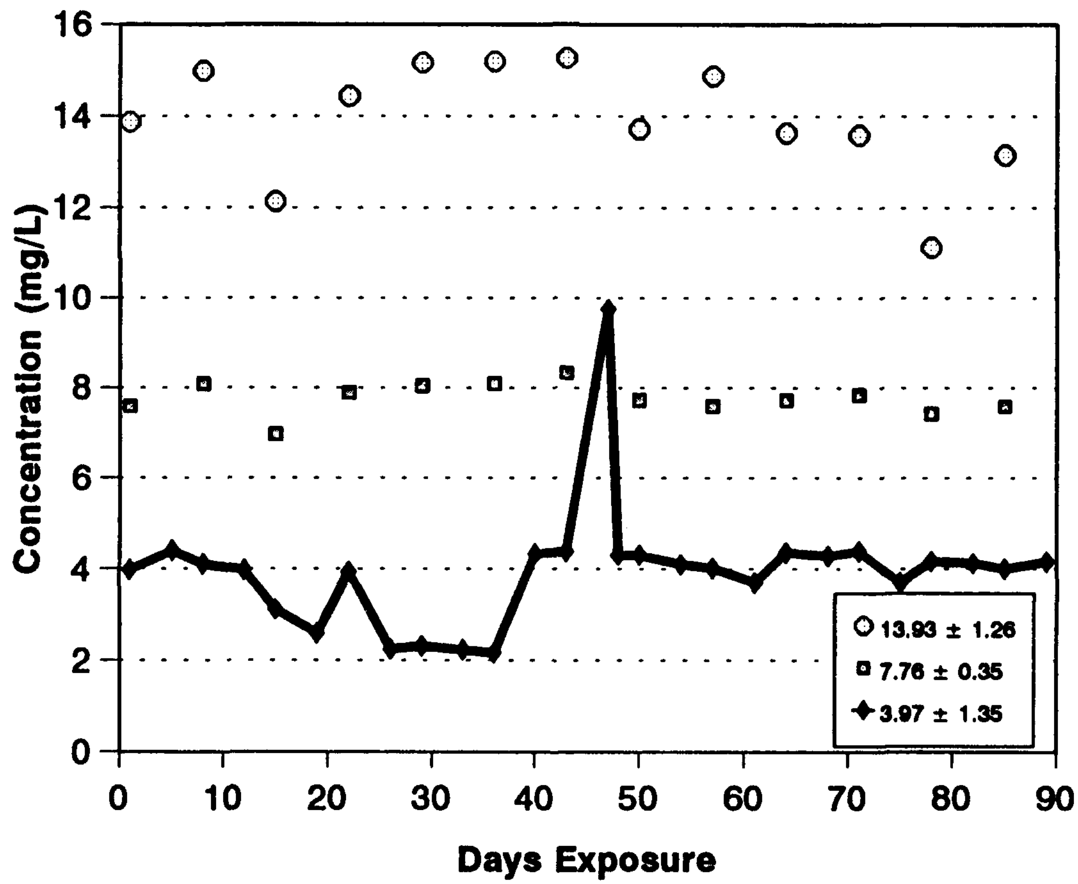

Figure 4.1. Mean measured TeCE concentrations in each of the 3 treatments during a 90-day medaka flow-through exposure. Two treatments received TeCE for a 24-hour period once a week. One intermittent treatment was at a nominal 15ppm, the other, 8ppm. The third treatment received a nominal 4ppm continuously. Overall mean values expressed as mean ± standard deviation.

○ One 24-hour (x 1) TeCE exposure each week at 15ppm

◘ One 24-hour (x 1) TeCE exposure each week at 8ppm

✦ Continuous TeCE exposure at 4ppm

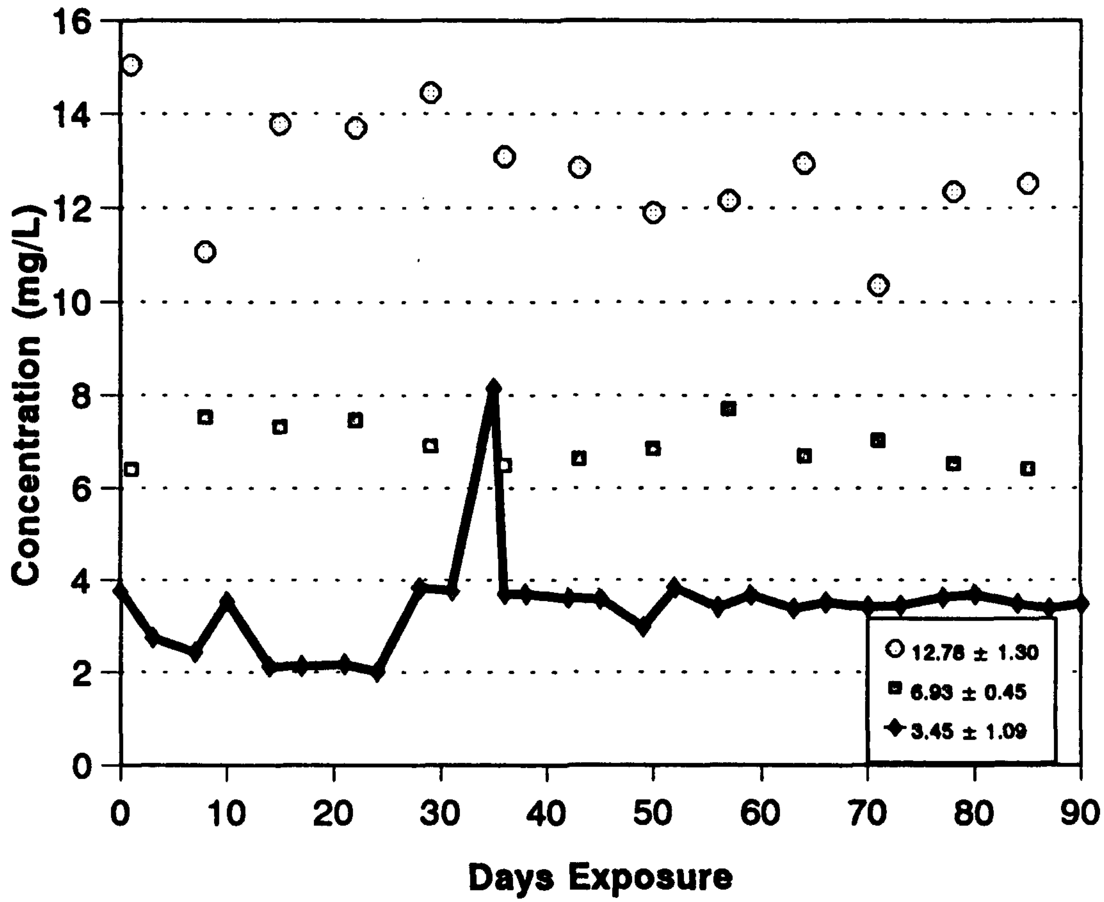

Figure 4.2. Mean measured TeCE concentrations in each of the 3 treatments during a 90-day guppy flow-through exposure. Two treatments received TeCE for a 24-hour period once a week. One intermittent treatment was at a nominal 15ppm, the other, 8ppm. The third treatment received a nominal 4ppm continuously. Overall values expressed as mean ± standard deviation.

○ One 24-hour (x 1) TeCE exposure each week at 15ppm

□ One 24-hour (x 1) TeCE exposure each week at 8ppm

◆ Continuous TeCE exposure at 4ppm

Table 4.1. Incidences of combined hepatocellular neoplastic lesions (altered focus, adenoma and carcinoma) in the medaka (*Oryzias latipes*) exposed to tetrachloroethane (TeCE).

Exposure Group	24 wk	36 wk	52 wk
Aq Control	0/73	1/75	0/68
Ft Control	1/72	NE	NE
4.0 TeCE	NE	NE	NE
Int. 8.0 TeCE	0/67	NE	NE
Int. 15.0 TeCE	0/75	1/76	1*/102

Aq= Aquarium
Ft= Flow-through
NE= Not Examined
Int.= Intermittent
*= a cholangiocellular neoplasm

Table 4.2. Incidences of combined hepatocellular neoplastic lesions (altered focus, adenoma and carcinoma) in the guppy (*Poecilia reticulata*) exposed to tetrachloroethane (TeCE).

Exposure Group	24 wk	36 wk	52 wk
Aq Control	NE	1/74	2/73
Ft Control	NE	NE	NE
4.0 TeCE	NE	NE	NE
Int. 8.0 TeCE	NE	NE	NE
Int. 15.0 TeCE	0/76	0/75	2/97

Aq= Aquarium
Ft= Flow-through
NE= Not Examined
Int.= Intermittent

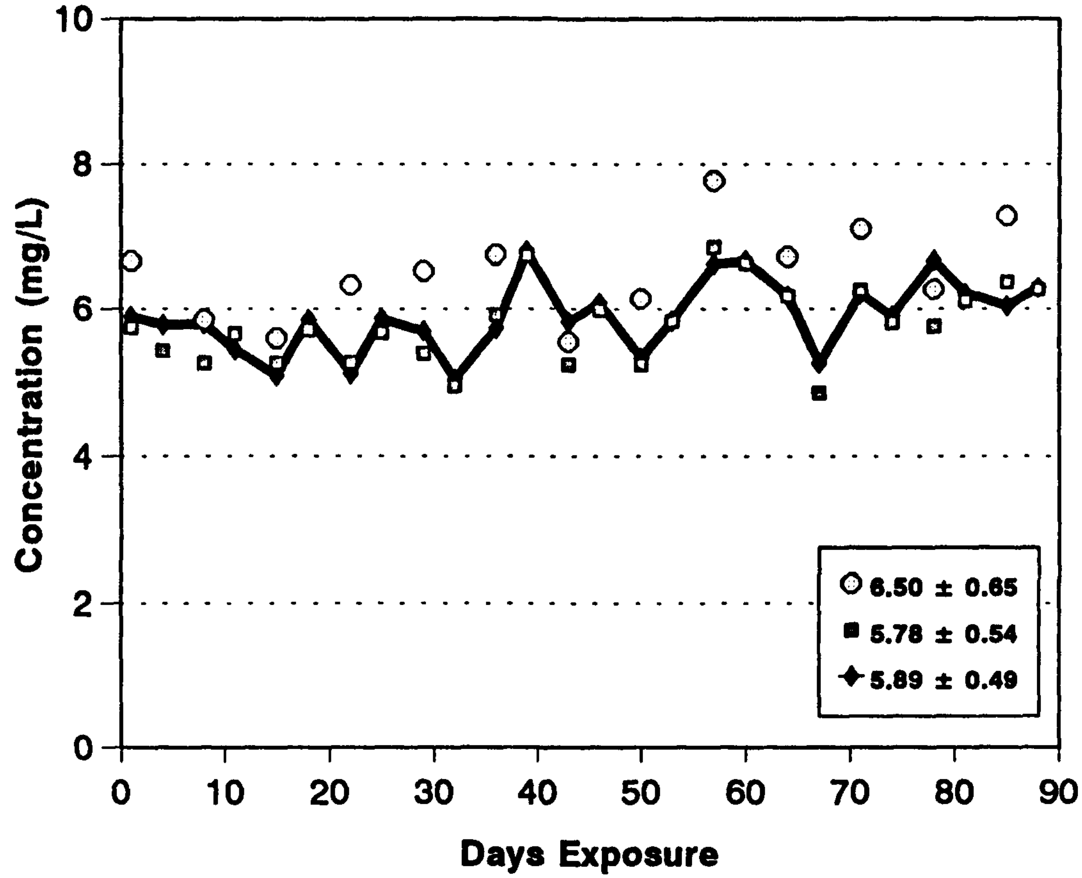

Figure 4.3. Mean measured CDBM concentrations in each of the 3 treatments during a 90-day medaka flow-through exposure. All 3 treatments received the same concentration of CDBM, either on a continuous or an intermittent basis. Overall mean concentrations for each treatment expressed as mean ± standard deviation.

○ One 24-hour (x 1) exposure each week
◻ Two 24-hour (x 2) exposures each week
◆ Continuous exposure to CDBM

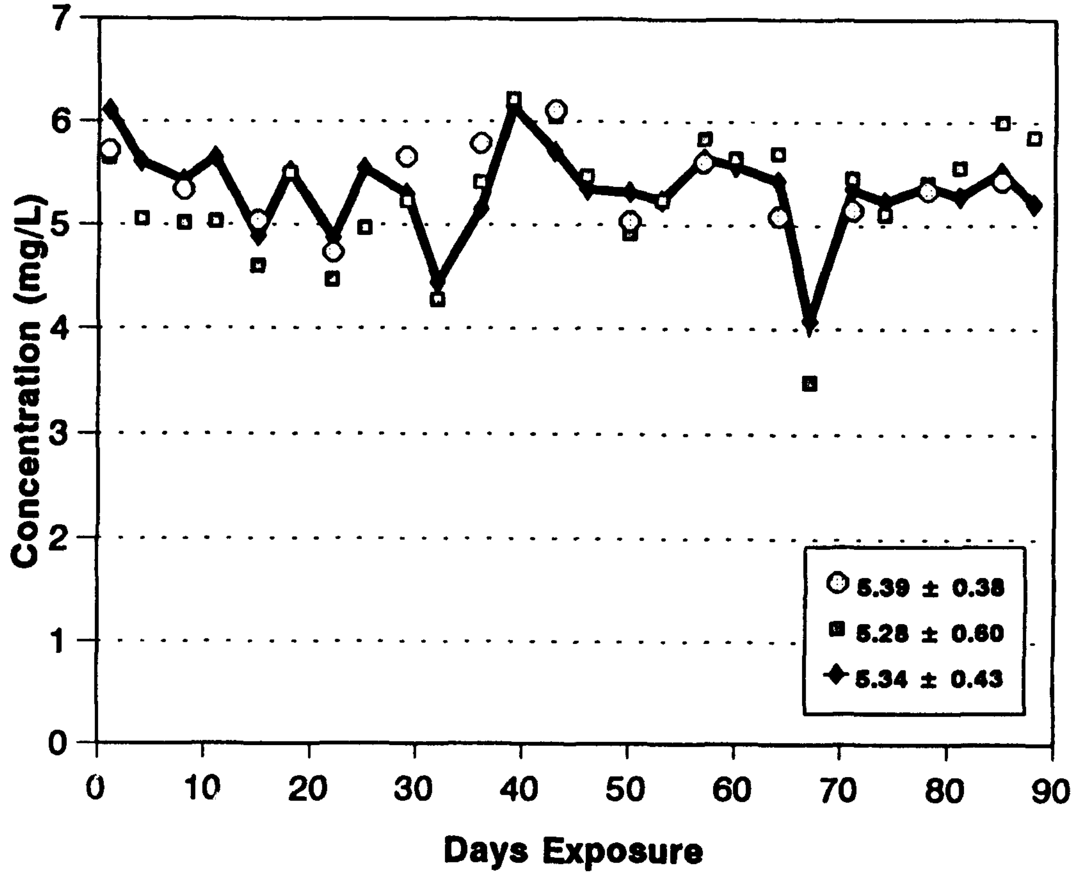

Figure 4.4. Mean measured CDBM concentrations in each of the 3 treatments during a 90-day guppy flow-through exposure. All 3 treatments received the same concentration of CDBM, either on a continuous or an intermittent basis. Overall mean concentrations for each treatment expressed as mean ± standard deviation.

○ One 24-hour (x 1) CDBM exposure each week

▪ Two 24-hour (x 2) CDBM exposures each week

◆ Continuous exposure to CDBM

Table 4.3. Incidence of hepatocellular neoplastic lesions (altered foci, adenoma, carcinoma) in medaka (*Oryzias latipes*) exposed to chlorodibromomethane.

Exposure group	**13 wk**	**24 wk**	**36 wk**
Aquarium control	0/5	0/73	1/72
Flow-through control	0/5	NE	NE
CDBM x 1	0/5	NE	NE
CDBM x 2	0/5	NE	NE
Continuous CDBM	0/5	0/76	0/42

NE= Not Examined

Table 4.4. Incidence of hepatocellular neoplastic lesions (altered foci, adenoma, carcinoma) in the guppy (*Poecilia reticulata*) exposed to chlorodibromomethane (CDBM).

Exposure group	**13 wk**	**24 wk**	**36 wk**	**52 wk**
Aquarium control	NE	0/72	0/75	0/15
Flow-through control	NE	NE	NE	NE
CDBM x 1	0/6	NE	NE	NE
CDBM x 2	0/6	NE	NE	NE
Continuous CDBM	0/6	0/66	1/67	0/18

NE=Not Examined

Discussion

The present study suggested that neither 1,1,2,2-tetrachloroethane (TeCE) nor chlorodibromomethane (CDBM) was carcinogenic to medaka and guppy when administered in flow-through exposures for 90 days with specimens examined up to 52 weeks post-initial exposure. There are several ways that this negative result could be interpreted. First, the specimens might not have been exposed long enough for the compound to elicit a carcinogenic response. This is unlikely in the present study because three months represents a considerable portion of the life span of the medaka and guppy. Furthermore, the high concentration exposures, at least, was administered at near the toxic limit.

Second, TeCE and CDBM might not have been carcinogenic to the medaka and the guppy because those fishes are not capable of metabolizing the compounds to their carcinogenic intermediates. TeCE has been shown to be hepatocarcinogenic in only one bioassay model, the B6C3F1 mouse, which is known to have a high spontaneous background of liver tumors (see Bolt, 1987). Because TeCE is considered highly hepatotoxic, at least to mammals, it is likely that recurrent hepatic damage and cell replication could have contributed to its carcinogenicity in the rodent model. Generally, chlorinated solvents are not as hepatotoxic in fish as they are in mammals. It also appears that hepatic microsomal metabolism by cytochrome P450-dependent mixed function oxidases plays a role in the carcinogenicity of TeCE as well as other chloroethanes (Ivanetich and van den Honert, 1981). Because numerous studies have shown that fish are incapable of metabolizing compounds like phenobarbital that require cytochrome P450, it may be that the medaka and guppy were not able to convert TeCE or CDBM to their carcinogenic intermediates because of those deficiencies.

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5.0. Carcinogenic effects of 1,2-dibromoethane on the medaka and changes in glutathione S-transferase activities

Introduction

1,2-Dibromoethane (DBE; ethylene dibromide) is a halogenated aliphatic hydrocarbon that has been used as a pesticide and gasoline additive and is of concern to humans because of potential industrial and environmental exposures (Brown, 1984; Hanson, 1984). In rodents, DBE induces neoplasms mainly at the site of exposure when administered chronically by gavage or inhalation (Weisburger, 1977; Olson et al., 1973; Wong et al., 1982). Although neoplastic lesions of the liver have been induced by DBE exposure (Wong et al., 1982; Moslen, 1984), the liver and other internal organs appear less sensitive than directly-exposed tissues. In rodents, DBE has an uncommon carcinogenic mechanism that utilizes what is normally a detoxification pathway, conjugation with glutathione, to form an electrophilic episulfonium ion species that alkylates DNA and initiates carcinogenesis (Guengerich et al., 1987).

The purpose of this study was to determine the carcinogenicity of DBE to the medaka (*Oryzias latipes*). This species as well as several others, especially the rainbow trout (Sinnhuber et al., 1977; Hendricks et al 1984), have received much recent attention as models for cancer research (Masahito et al., 1988). Medaka have been shown to be sensitive to several carcinogens (Couch and Harshbarger, 1985), developing neoplastic lesions mainly in the liver following exposure to such compounds as methylazoxymethanol acetate (Aoki and Matsudaira, 1984; Hawkins et al., 1985, 1988; Hinton et al, 1986;), diethylnitrosamine (Kyono-Hamaguchi, 1984; Ishikawa et al., 1979), benzo[a]pyrene (Hawkins et al, 1988), acetylaminofluorene and aflatoxin (Hatanaka et al., 1982). In this study, we examined the carcinogenicity of DBE to medaka exposed under flow-through conditions. We also examined the effects of DBE exposure on hepatic GSH content and GSH-S-transferase activity in medaka.

Materials and methods

Fish. Medaka were produced from laboratory-reared broodstock cultured at the Gulf Coast Research Laboratory since 1982 following protocols described above in section *3.0. Methodology*. Upon hatching, test fish were fed nematodes three times daily and paramecium once a day for the first two days. At three days post-hatch they were fed artemia twice a day and nematodes and paramecium once daily. Fish were 6- to 10-days old when they entered the test system. During exposure fish were fed a diet consisting of artemia nauplii 48-hours post-hydration and a commercial diet (Aquavet Stress Formula Flake). At the end of the approximately three-month exposure period, fish were moved to static grow-out aquaria containing noncontaminated water maintained at 26 ± 1°C. Fish were fed a commercial diet (AquaVet Stress Formula Flake) supplemented with live brine shrimp daily. Progeny of test fish were removed from the exposure systems. Moribund specimens were removed and processed as they were identified.

Exposures. The carcinogenicity studies incorporated five test groups that included the following: 1) aquarium control, 2) flow-through control, 3) low concentration exposure, 4) intermediate concentration exposure, and 5) high concentration exposure. The aquarium control group was held in 50 L aquaria equipped with sponge filters and containing toxicant-free water. The flow-through control group as well as the three DBE exposure groups were held in an enclosed continuous-flow apparatus described by Walker et al. (1985). Flow rates were adjusted to provide six volume additions each 24 hours.

Selection of exposure concentrations was based on the results of preliminary range-finding tests. DBE concentrations were measured in the ambient aquarium water by gas chromatography. Nominal concentrations for the carcinogenicity studies were 0.1 mg/L, 10.0 mg/L, and 20 mg/L, representing low, intermediate, and high concentration groups, respectively. Exposures at the nominal 0.1 mg/L and 10.0 mg/L (low and intermediate concentration groups) were continuous. Preliminary toxicity studies, however, showed that continuous exposure to a nominal 20 ppm DBE (high) concentration was lethal. Therefore, this concentration was administered intermittently for 24 hours once a week for the approximately three month study.

Specimens were assigned to treatment aquaria using a computer-generated random number method as described above in section *3.6. Randomization and sampling*. On the initial day of the exposure, the number of specimens in each exposure aquarium was reduced to 300. Mortalities up to that time were taken into account in order to calculate the number of fish to be removed. On day 78, filter sponges were added to each aquarium as egg deposition substrates. Toxicant flow to the 10 ppm treatment aquarium (intermediate concentration) was stopped on day 79 because of excessive mortality and samples were taken for histopathology. All exposures were terminated on day 103. Fish were removed by gentle netting from the exposure aquaria. Specimens were divided into replicates of 90-100 specimens and relocated in grow-out aquaria containing toxicant-free water and equipped with sponge filters. One replicate from each exposure was photographed for length determinations prior to transfer to grow-out.

Pathology. Samples for histopathology were collected at 24, 36, and 58 weeks from the beginning of exposure. Fish were anesthetized with tricainemethanesulfonate (MS-222), examined grossly, and fixed in Lillie's solution (10% neutral-buffered formalin: picric acid: formic acid). The abdominal cavities of large fish were usually opened to facilitate fixation of the viscera. Whole fish were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Specimens were embedded in paraffin blocks so that longitudinal sections through the dorso-ventral plane could be obtained. Each specimen was sectioned and examined in two planes, one about sagittal and one about median. This sectioning protocol allowed us to examine the liver and most other major organs including kidney, gill, intestine, exocrine pancreas, gonad, eye, brain and spinal cord, pseudobranch, thyroid gland, swimbladder, and heart.

GSH-S transferase activity. Adult medaka of both sexes were exposed under flow-through conditions as described above to a nominal concentration of 1.0 ppm DBE for 14 to 38 days. Fish were removed to clean water for 24 hours, then sacrificed. Livers from 20 to 22 control fish and 19 to 23 DBE-exposed fish were removed for each pool. In one experiment, one pool of treated and one pool of control fish were studied, whereas in two subsequent studies, 3 pools each of treated and control fish were used. The liver pools were weighed, homogenized in 5 volumes of 1.15% KCl/0.05 M potassium phosphate (pH 7.4) and centrifuged at 13,000 x g for 20 minutes. The 13,000 x g supernatant was centrifuged at 170,000 x g for 45 minutes and the supernatant fraction (cytosol) used in assays. GSH-S transferase activity with 1-chloro-2,4-dinitrobenzene was measured spectrophotometrically at 25°C as described by James et al (1979). The protein content of cytosol was determined by the Lowry method (1951). The GSH content of cytosol was measured by a modification of the method of Hissin and Hilf (1976).

Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of cytosol samples was performed as described by Laemmli (1970) on a 7.5% acrylamide gel. The gels were stained with Coomassie Blue.

Statistical Method. Analysis of tumor incidence data was made by Fisher's Exact Test. Incidences of tumor types were compared against combined (aquarium and flow-through groups) control incidences for specific sample times.

Results

Exposure concentrations. DBE concentrations measured during the study are shown in Figure 5.1. Traces of DBE, <0.035 mg/L, were measured in the flow-through control aquaria. The low concentration exposure, nominally 0.1 mg/L, was administered continuously and actually measured 0.133 ± 0.020 mg/L. The intermediate concentration, also administered continuously, was nominally 10.0 mg/L. Because of excessive mortality, this concentration was reduced by 25% on day 33 of the exposure and again on day 46 by an additional 25%. Over the course of the exposure, this concentration averaged 6.2 ± 2.48 mg/L. The high concentration, nominally 20 mg/L, was administered for 24 hours once a week for 13 weeks. Those concentrations measured during the exposure averaged 18.58 ± 1.55 mg/L.

Toxic responses. Table 5.1 summarizes toxic responses in medaka during the exposure period. Mortalities in the intermediate exposure group were excessively high, apparently because of DBE toxicity. An unexplained mortality of 6%, however, occurred in the flow-through control group. Fecundity, as measured by the total number of viable eggs produced during a 23-day collection period was severely affected in all DBE exposure groups, most severely in the intermediate exposure group in which no viable eggs were produced. Furthermore, 6.8% of the embryos produced in the low concentration group showed some kind of gross abnormality.

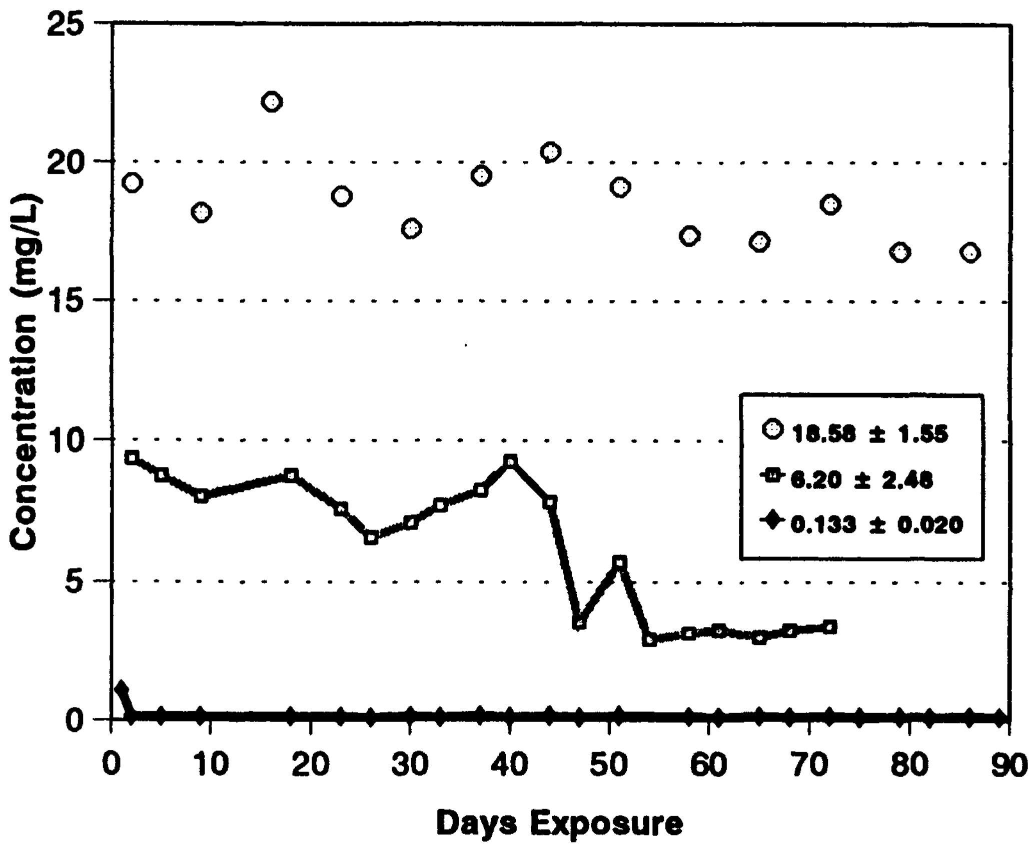

Figure 5.1. Mean measured DBE concentrations in each of the three treatments during a 90-day medaka flow-through exposure. The high concentration was administered for a 24-hour period once each week. The intermediate and low exposures were to be continuous, one at a nominal 10ppm and the other at 0.1ppm. However, due to excessive mortality in the intermediate concentration, DBE levels were incrementally lowered beginning on day 43, and discontinued on day 72. Overall mean values expressed as mean ± standard deviation.

○ One 24-hour (x 1) DBE exposure each week

▫ Continuous DBE exposure at intermediate concentrations to day 72

◆ Continuous DBE exposure at 0.1ppm

Table 5.1. Toxic responses in medaka during 90-day DBE exposure period[1]

	Measured DBE Concentrations (mg/L)				
Parameter	**Aq Ctl**	**Ft Ctl**	**0.13**	**6.20**	**18.6[2]**
Mortality	1.7%	6.0%	0.3%	46%	1.1%
Fecundity[3]	114	229	59	0	2
Viable Embryos (%)	91.9	89.5	98.3	--	100
Hatch (%)	54.4	44.1	43.3	--	0
Fry survival (%)[4]	91.9	84.2	53.8	--	--
Abnormal Embryos (%)	0	0	6.8	--	0

[1]Fry were 7 days old upon initiation of exposures
[2]Thirteen 24-hr intermittent exposures at weekly intervals
[3]Total number of viable eggs collected over a 12-day period
[4]Fry surviving 72 hours post hatch

Pathology. Table 5.2 shows the incidences of hepatocellular neoplastic lesions, cholangiolar neoplasms, gall bladder neoplasms and thymic lymphoma in specimens in the test groups. Specimens dying at other than scheduled sampling were included in the closest sampling period. Only one neoplastic hepatic lesion was found in a control group specimen, a hepatocellular adenoma in a specimen from the aquarium control group from the 36 week sample. This brought the total incidence of hepatocellular neoplastic lesions in combined control groups to 0.33% (1/299). A second neoplasm, which occurred in a flow-through control specimen from the 36-week sample, was a poorly-differentiated acinar cell carcinoma of the exocrine pancreas, a neoplasm considered to be spontaneous (Hawkins et al., 1991). Thus, the combined incidence for all neoplasms from both control groups was 0.67% (2/299).

DBE induced statistically significant incidences of hepatocellular and cholangiolar neoplasms in specimens from the intermediate and high DBE exposure groups. Gall bladder neoplasms occurred in statistically significant incidences only in the high concentration exposure. Medaka did not often appear to die from neoplasms although some specimens that became moribund and were killed before scheduled sampling periods had neoplasms on histological examination. The earliest neoplastic lesion was a hepatocellular carcinoma that occurred in a specimen from the intermediate exposure group at 17 weeks.

Hepatocellular neoplastic lesions included those categorized as persistent foci of cellular alteration (altered foci), adenomas, and hepatocellular carcinomas. Altered foci occurred in 58-week specimens of both the intermediate and high concentration groups. Altered foci were spherical areas staining hyperchromatically, that is, basophilic or eosinophilic. The lesions consisted of well-differentiated cells that usually blended with neighboring unaffected cells. Hepatocellular adenomas occurred in 32% of the specimens from the high concentration group in the 24 week sample. Adenomas were dense, spherical lesions consisting almost exclusively of basophilic, well-differentiated hepatocytes having a low mitotic index. Cells of adenomas were distinctly different from non-neoplastic hepatic parenchymal cells in their inability to store cytoplasmic fat and glycogen. Also categorized as adenomas were several proliferative lesions consisting of large cells that stained lightly compared to normal cell and appeared to grow outward and compress adjacent normal tissues.

Hepatocellular carcinomas occurred in low incidence in the 36 week sample from the intermediate concentration group and were the predominant hepatocellular neoplasm in specimens from the high concentration group in which they occurred in 50% of the specimens from the 24 week sample and were often extensive lesions involving most of a liver. Hepatocellular carcinomas were distinguished from adenomas by their larger size, greater tendency to be undifferentiated, invasiveness, and high mitotic index. Well differentiated hepatocellular carcinomas were composed almost exclusively of basophilic cells that closely resembled hepatocytes whereas in poorly differentiated carcinomas the neoplastic hepatocytes often were irregular or spindle shaped.

Cholangiolar neoplasms which included cholangiomas and cholangiocarcinomas occurred mainly in specimens from the high concentration exposure, especially in the 36- and 58-week samples. Cholangiomas were composed mainly of well-differentiated ducts lined by a single layer of flattened, cuboidal or columnar epithelial cells that had basally-situated nuclei. Few mitotic figures were noted in these lesions and the lesions did not appear to invade normal tissues. By comparison, cholangiocarcinomas were extensive lesions of irregularly formed ducts that showed intrapapillary development and were lined by epithelial cells that were frequently stacked and pleomorphic. Mitotic activity was greater in cholangiocarcinomas than in cholangiomas and cholangiocarcinomas appeared to actively invade normal tissues.

Neoplasms that appeared to originate from the gall bladder epithelium or from ducts near their entrance into the gall bladder occurred both within the liver and in the peritoneal cavity where they sometimes caused the gall bladder to become nearly the size of the liver. These lesions were clearly distinct from other lesions classified as cholangiocellular neoplasms. Gall bladder neoplasms consisted mainly of well-differentiated tall columnar epithelial cells that had basally-situated, usually elongate, nuclei. At the cell apices, small pyramidal projections extended into the lumen. Lumens often contained what appeared to be

Table 2. **Incidences of specific neoplastic lesions in Japanese medaka exposed to 1,2-dibromoethane (DBE)**

	Controls (aquarium and flow-through)			0.133 DBE			6.20 DBE			18.58 DBE		
Lesions	24 wks	36 wks	58 wks	24 wks	36 wks	58 wks	24 wks	36 wks	58 wks	24 wks	36 wks	58 wks
Hepatocellular												
Altered foci	0/62	0/112	0/125	0/31	0/49	0/60	0/24	0/50	3/20**	0/28	0/78	4/66*
Adenoma	0/62	1/112	0/125	1/31	1/49	0/60	2/24	2/50	3/20**	9/28**	2/78	4/66*
Carcinoma	0/62	0/112	0/125	0/31	1/49	0/60	0/24	6/50**	0/20	14/28**	15/78**	15/66**
Cholangiolar												
Cholangioma	0/62	0/112	0/125	0/31	1/49	0/60	1/24	3/50*	0/20	0/28	3/78	3/66*
Carcinoma	0/62	0/112	0/125	0/31	0/49	0/60	0/24	2/50	1/20	1/28	4/78*	4/66*
Gall bladder	0/62	0/122	0/125	0/31	0/49	0/60	1/24	0/50	1/20	0/28	3/78	6/66**
Lymphoma	0/62	0/112	0/125	0/31	0/49	1/60	0/24	3/50*	1/20	1/28	1/78	0/66

* Statistically significant from parallel control incidence by Fisher's Exact Text, P<0.05
** Statistically significant from parallel control incidence by Fisher's Exact Test, P<0.01

solidified bile. The cells were usually arranged in papillae which often bridged between one another forming pseudoductules. Neoplastic cells were not observed to breach the basal lamina. Mitotic figures were not frequently observed.

DBE also appeared to increase the incidence of thymic lymphoma (lymphoblastic lymphoma, lymphosarcoma) in exposed specimens. As described earlier (Battalora et al., 1990), the lymphomas appeared to originate in the thymus located in the posterodorsal fornix of the gill chamber. Tumor cells were uniformly small lymphocytic cells with a high nucleus-to-cytoplasm ratio and frequent mitotic figures. Tumor cells invaded adjacent muscle tissue, spaces in the head region, the kidney, and the peritoneal cavity.

GSH-S-transferase activity. Specimens in the first group were exposed to 1.3 ppm DBE. The GSH-S-transferase activity of hepatic cytosol from DBE-exposed medaka was more than double that of controls and the GSH content of liver was also higher in treated fish than in controls (Table 5.3).

Table 5.3. Hepatic GSH content and GSH-S-transferase activity in medaka exposed for 14 days to 1.3 ppm DBE.

	GSH-S-T[a] µmole/min/mg protein	GSH Content[b] mM
Control	1.5	1.5
Treated	3.56	2.1

[a]With 1mM 1-chloro-2,4-dinitrobenzene as substrate, in the presence of 5 mM added GSH.
[b]Assayed by the method of Hissin and Hilf (1976).

SDS-PAGE of the cytosol fractions showed that DBE-exposed medaka had a pronounced increase in a band at 26,000 daltons. In subsequent experiments the actual DBE concentration was 1.0 ppm. Since separate pools were studied, it was possible to determine the statistical significance of the effect of DBE exposure on GSH-S-transferase activity. For both 14 and 38 day exposures, hepatic cytosol from pools of DBE- exposed medaka had significantly higher ($p<0.05$) GSH-S-transferase activity than controls. GSH concentration was not determined in these studies.

Discussion

Medaka appear more sensitive to the carcinogenic effects of DBE than any other organism tested and the liver and gall bladder are the primary target organs. In samples taken 24 weeks from the beginning of a 79-day exposure, almost 20% of specimens from the intermediate concentration exposure (6.20 ppm DBE) had neoplastic lesions of the liver, gall bladder, or both. In specimens from the high concentration exposure (exposed to about 18.58

ppm DBE for 24 hours once each week for 103 days), more than 64% had liver and gall bladder neoplasms. The incidence of the neoplasms in both control groups at the 24 week sampling period was 0% whereas the overall control incidence of hepatic and gall bladder neoplastic lesions for the 24, 36 and 58 week sampling periods was 0.33% (1/299).

DBE, along with 1,2-dibromo-3-chloropropane, were the most carcinogenic halogenated compounds tested by oral intubation in two year studies on rats and mice. Those compounds caused mainly squamous cell carcinomas in the stomachs of both those species (Olson et al 1973; Weisburger, 1977). When administered by chronic inhalation, DBE induced proliferative and various neoplastic lesions in the nasal cavities, circulatory system and lungs of rats and mice (Stinson et al., 1981; NTP, 1981; and Reznik, 1980).

In rodents as compared with the medaka, the liver does not appear to be a primary target organ of DBE. Wong et al. (1982), however, reported high percentages of internal neoplasms including those of mammary gland, spleen, adrenal gland, liver, kidney, and subcutaneous tissues in rats receiving DBE by inhalation for 18 months. Addition of disulfuram, a drug used in the treatment of alcoholism, increased tumor incidences, especially those of hepatocellular tumors.

Application of elaborate short-term initiation-promotion protocols to induce altered liver foci often show hepatocarcinogenic effects induced by non-hepatocarcinogens (Pereira, 1982). Rats given one or two oral doses of DBE followed by partial hepatectomy then a second hepatectomy 90 days later, promoted by phenobarbital in the drinking water for four months and terminated at 16 months had up to 3 times (25 out of 41) the incidence of hepatocellular/nodules as the controls (Moslen, 1984). Hepatocarcinogenic initiation did not occur in rats given DBE by gavage once (120 mg/kg) or four times (60 mg/kg), partially hepatectomized, then promoted by 500 ppm phenobarbital in the drinking water, and sacrificed at 67 days (Milks et al., 1982). Applying the Solt-Farber selection procedure (Tsuda et al., 1980), Moslen et al. (1984) initiated hepatocarcinogenesis in rats given a single oral dose of DBE, partial hepatectomy 4 hours later, five oral doses of AAF, a single IP dose of carbon tetrachloride, a booster dose of AAF and sacrificed on day 82. Hepatocarcinogenic effects occurred in 4 of 6 rats tested and none in the controls.

In medaka, DBE induced hepatocellular, cholangiocellular, and gall bladder neoplasms in relation to dose and time post-exposure. Although the incidence of lymphosarcoma was increased in some DBE exposure groups, we do not believe that this study provides sufficient evidence that the lesion was DBE-induced. In other carcinogen bioassays in our laboratory, lymphosarcoma has occurred infrequently and in low numbers in both exposed and control specimens (Battalora et al., 1990).

Three types of carcinogen-induced hepatocellular lesions were recognized including altered foci, hepatocellular adenomas, and hepatocellular carcinomas. Diagnostic criteria and nomenclature were originally based on descriptions of carcinogen-induced hepatocellular neoplasms in rainbow trout (Hendricks et al., 1984) and applied in other carcinogen bioassays on medaka and guppy (Hawkins et al., 1988a, b). These lesions correlate highly with carcinogen exposure in fish regardless of species or carcinogen tested, or route of exposure (Couch and Harshbarger, 1986; Hawkins et al., 1988). Indeed, the liver appears to be the most sensitive target organ of most fish carcinogens in fish bioassays. Fish models thus resemble rodent initiation-promotion models in identifying by way of hepatic effects both liver and non-liver carcinogens. The fact that immersion exposure of medaka by DBE induced liver neoplasms and no external or site-of-contact neoplasms occurred is consistent with previous studies in fish in which superficial neoplasms were infrequently observed or did not occur at all. Although the gill is a likely site of tumor development because of its direct contact with environmental toxicants and transport functions, gill tumors rarely occur in fishes. Low incidences of gill neoplasms in medaka have been reported following exposure to only a few compounds, for example benzo(a)pyrene (Hawkins et al., 1988) and MNNG (Britelli et al., 1985).

The carcinogenic mechanisms of DBE are unusual. DBE stimulates mitosis in rat liver (Nachtomi and Farber, 1978) where it also causes DNA damage (Storer and Conally, 1983; Nachtomi and Sarma, 1977). The mitogenic stimulus and DNA damage, however, apparently do not enhance carcinogenesis. Ledda-Columbano et al. (1987) compared liver cell mitogenesis induced by DBE with compensatory cell proliferation induced by necrogenic treatments of carbon tetrachloride and found that carcinogenesis after post-treatment with diethylnitrosamine was enhanced in those treated with carbon tetrachloride (necrogenic treatment) but not in those treated with DBE (mitogen treatment). An important route of activation of DBE is GSH conjugation which yields an electrophilic episulfonium ion that can alkylate DNA and RNA (Guengerich et al. 1987). The GSH-S-transferases that catalyze the reaction of DBE and GSH belong to a family of enzymes found mainly in the cytosol but also in other subcellular fractions of liver and extrahepatic organs (Armstrong, 1991). The GSH-S-transferase enzymes consist of homo or heterodimers of polypeptide chains with molecular weight 23-28,000 daltons (Mannervik and Danielson, 1988). Although there is substrate selectivity among members of the GSH-S-transferase family, all known forms are reactive towards 1-chloro-2,4-dinitrobenzene. It was interesting that medaka responded to low levels of DBE exposure by increasing GSH-S-transferase activity towards 1-chloro-2,4-dinitrobenzene, and that a protein with molecular weight 26,000 daltons was increased in exposed fish. Untreated medaka have low hepatic GSH-S-transferase activity, compared to other small fish species (James et al., 1988). The present study suggests that the ability of medaka to metabolically activate DBE to a reactive metabolite will increase over control levels with continued exposure. It remains to be determined whether the DNA adduct that leads to the genetic damage in medaka is the same as the one in rodents (Guengerich et al., 1987).

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6.0. Induction of hepatic neoplasia in guppy and medaka by vinylidene chloride

Introduction

Vinylidene chloride (1,1-dichloroethylene), an air and drinking water contaminant (U.S. EPA, 1982,1986), is a monomer used to produce polyvinylchloride resins and plastic food wraps (Quast et al., 1986). Its potential for workplace and consumer exposure and its structural similarity to other compounds of environmental interest such as vinyl chloride, trichlorethylene, 1,2-dichloroethane, 1,2-dibromoethane, and tetrachloroethylene make vinylidene chloride (VC) a likely candidate for carcinogen bioassay. Recently, the International Agency for Research on Cancer (IARC) has reevaluated VC and concluded that it, along with trichloroethylene, belongs to the Group 3 animal carcinogens, meaning that there is limited evidence for the carcinogenicity of those compounds to animals (Van Duuren, 1989). The term "limited evidence" is applied because whereas two-year bioassays of Fisher 344 rats and B6C3F1 mice did not result in significantly increases in tumors of dosed as compared with control animals (see Chu and Milman, 1981), an inhalation study on Swiss mice resulted in increased incidences of kidney tumors in the male specimens (Maltoni et al., 1977; Maltoni, 1977).

We conducted carcinogenesis bioassays with VC against the medaka and the guppy in flow-through exposures. Histopathological examination of specimens exposed to VC for three months then grown-out in clean water for additional periods of three, six, or nine months indicated that this compound is hepatocarcinogenic to both species. Although the incidences are in the range of 15-20%, which we consider a moderate carcinogenic response in these models, the lesions were histologically well-advanced and would certainly affect the health of the organisms.

Materials and Methods

Medaka and guppy were exposed under flow-through conditions for 3 months to VC (Aldrich Chemical Company; 99% purity). Following a series of range-finding tests, a targeted exposure concentration of 40 ppm was chosen. Three hundred 6 to 7-day post-hatch medaka and 300, 24 to 48-hour old guppies were assigned to control and treatment aquaria using methods described above in *3.6. Randomization and sampling.*

The tests incorporated the following treatment groups:

1. Aquarium control group (situated outside the exposure system)

2. Flow-through control group (situated inside the exposure system and thus subject to low levels of volatile test compounds)

3. An exposure group in which a nominal 40 mg/L VC concentration was delivered continuously for the 90-day exposure

4. An exposure group to which a nominal 40 mg/L VC concentration was delivered once for 24 hours each week (1X group)

5. An exposure group in which a nominal 40 mg/L VC concentration was delivered twice for 24 hours each week (2X group)

The test compound was delivered to the exposure chambers under conditions and using methodology described above in section *3.2. Exposure techniques*. In this study, the test groups were organized similar to the chlorodibromomethane study described above in section 4.0.

Results

VC values measured by gas chromatography are depicted in Figures 6.1 and 6.2 for the medaka and guppy exposures, respectively.

Several studies aimed at determining the acute and chronic toxicological effects of VC on medaka and guppy were conducted. At day 28, fifteen randomly sampled fish from each retention chamber were fixed for histopathological and electron microscopical analyses. Another 25 fish from each retention chamber were examined for compound-related growth reduction. Each fish was measured to the nearest millimeter standard body length, and individual wet and dry weights obtained. A one-way analysis of variance, followed by a Newman-Keuls multiple comparison test, was applied to this data for each species. Results presented in Table 6.1 indicate that intermittent exposure to 40ppm VC did not affect the growth of young medaka.

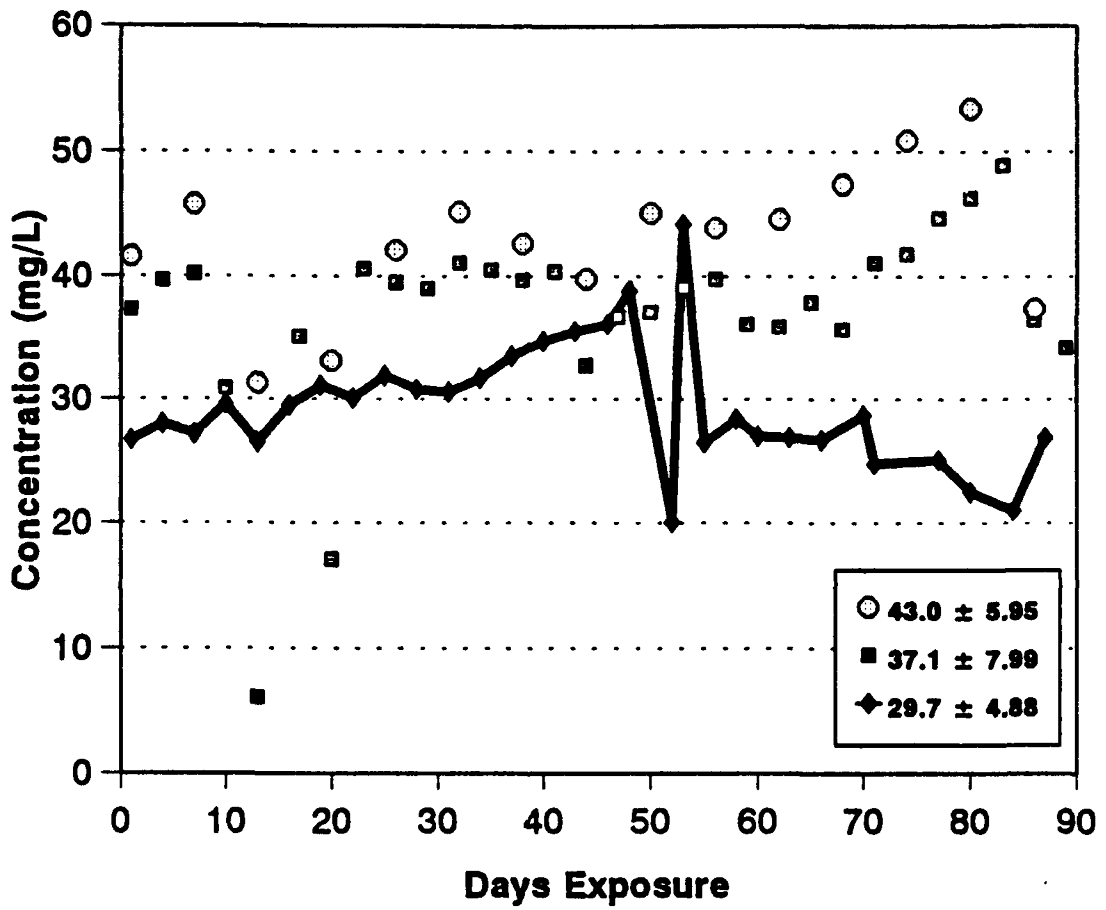

Figure 6.1. Mean measured VC concentrations in each of the three treatments during a 90-day guppy flow-through exposure. Two treatments received a nominal 40ppm on an intermittent basis; one, once every 6 days, the other, twice every 6 days. The third treatment received 30ppm on a continuous basis. Overall mean concentrations for each treatment are expressed as mean ± standard deviation.

○ One 24-hour (x 1) VC exposure every 6 days

▣ Two 24-hour (x 2) VC exposures every 6 days

◆ Continuous VC exposure

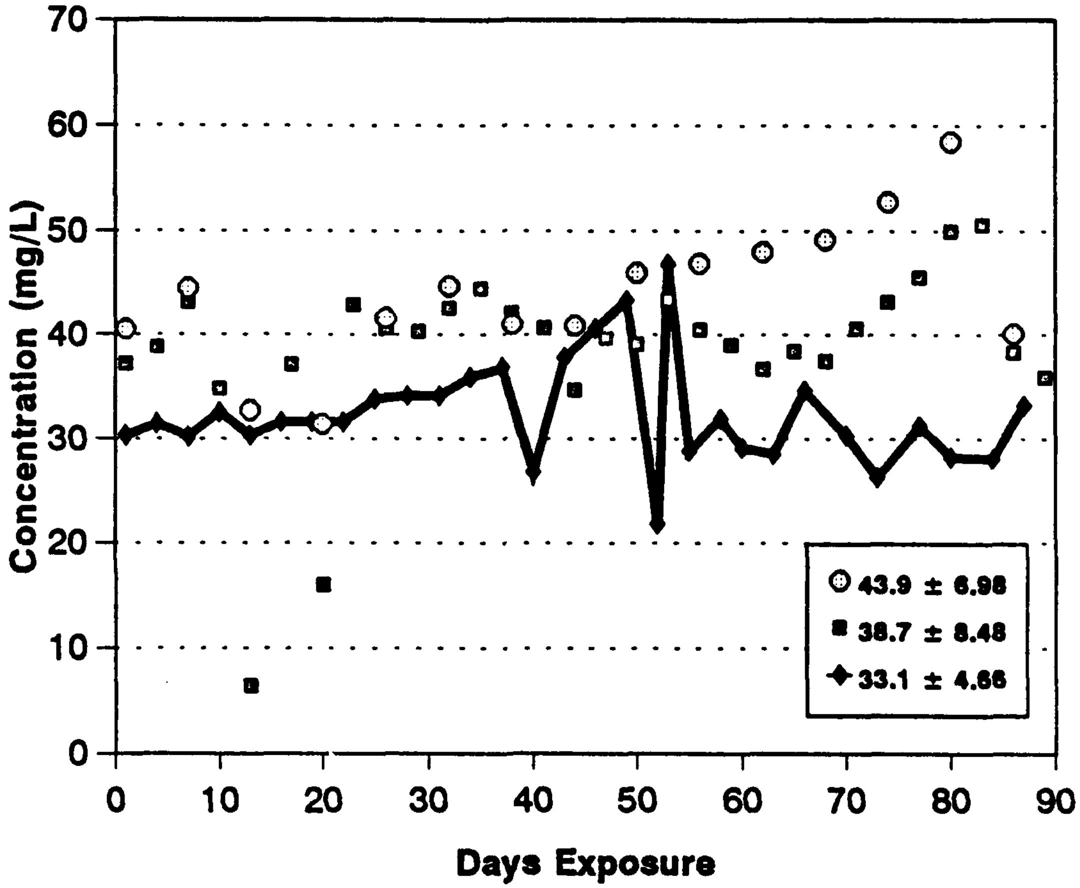

Figure 6.2. Mean measured VC concentrations in each of the three treatments during a 90-day medaka flow-through exposure. Two treatments received a nominal 40ppm on an intermittent basis; one, once every 6 days, the other, twice every 6 days. The third treatment received 30ppm on a continuous basis. Overall mean concentrations for each treatment are expressed as mean ± standard deviation.

○ One 24-hour (x 1) VC exposure every 6 days

▫ Two 24-hour (x 2) VC exposures every 6 days

◆ Continuous VC exposure

Table 6.1. Mean body measurements of medaka from retention chambers at day 28 of exposure to vinylidene chloride (VC).

	Length (mm)	Wet Weight (mg)	Dry Weight (mg)
Aq. Control	12.3^{1*}	22.9^{1}	5.9^{1}
Ft. Control	11.4^{1}	17.4^{1}	4.5^{1}
1 x VC	11.9^{1}	24.5^{1}	5.3^{1}
2 x VC	12.0^{1}	23.2^{1}	5.5^{1}

*Within each category, treatment means with different numbers were determined to be statistically different at the 0.01 level

However, as results in Table 6.2 suggest, intermittent exposure to 40ppm VC significantly retarded the growth (length, wet weight, and dry weight) of guppies.

Table 6.2. Mean body measurements of guppy from retention chambers at day 28.

	Length (mm)	Wet Weight (mg)	Dry Weight (mg)
Aq. Control	12.0^{2*}	30.5^{2}	$6.7^{2,3}$
Ft. Control	12.2^{2}	31.2^{2}	8.0^{3}
1 x VC	11.2^{1}	25.0^{1}	5.5^{2}
2 x VC	10.8^{1}	22.2^{1}	3.8^{1}

*Within each category, treatment means with different numbers were determined to be statistically different at the 0.01 level

At the termination of the exposures, fish were removed to grow-out aquaria to be sampled at 24, 36, and 52 weeks from the time they entered the exposure system. Results of histopathological analyses are summarized in Tables 6.3 and 6.4.

Table 6.3. Incidence of hepatocellular neoplastic lesions (altered focus, adenoma and carcinoma) in the medaka (*Oryzias latipes*) exposed to vinylidene chloride (VC).

Exposure Group	24 wk	36 wk	52 wk
AQ CTL	0/102	0/74	1/99
FT CTL	0/77	0/75	0/80
x1 VC	1/89	4/74	4/74
x2 VC	2/91	11/70	5/57
40ppm cont. VC *	2/88	2/30	1/23
30ppm cont. VC	10/85	6/74	14/59

*Exposure was interrupted after 2 weeks due to excessive mortality and specimens removed to grow-out; a new exposure at 30ppm VC was initiated and continued for 90 days

Table 6.4. Incidence of hepatocellular neoplastic lesions (altered focus, adenoma and carcinoma) in the guppy (*Poecilia reticulata*) exposed to vinylidene chloride (VC).

Exposure Group	24 wk	36 wk	52 wk
AQ CTL	0/71	0/79	0/85
FT CTL	0/90	1/73	1/90
x1 VC	1/72	2/75	8/76
x2 VC	8/65	4/74	9/96
40ppm cont. VC *	22/77	1/31	2/24
30ppm cont. VC	12/75	8/76	6/49

*Exposure was interrupted after 2 weeks due to excessive mortality and specimens removed to grow-out; a new exposure at 30ppm VC was initiated and continued for 90 days

Discussion

These studies indicate that vinylidene chloride (VC) is carcinogenic to both medaka and guppy when administered in flow-through exposures for 90 days with specimens examined at 24, 36, and 52 weeks post-initial exposure. In the guppy, incidences of hepatocellular neoplastic lesions (total of altered foci, adenomas, and carcinomas) in the high concentration (30ppm continuous 90-day exposures) was statistically significant. In similarly conducted tests, we have examined the carcinogenicity of two other compounds, 1,1,2,2-tetrachloroethane (TeCE), 1,2-dibromoethane (DBE), and trichloroethylene (TCE) that are structurally similar to VC. Neither TeCE or TCE was carcinogenic to the guppy or medaka whereas DBE was highly carcinogenic to the medaka. We have not yet tested DBE in the guppy. The metabolic activation of DBE involves conjugation with glutathione, a pathway normally associated with detoxification. Although the metabolism of VC in fish has not been examined, in rodents VC is metabolized by mixed function oxidases to an unstable epoxide that can form chloroacetyl chloride, a highly reactive intermediate which can be detoxified by glutathione, bind to macromolecules, or be further metabolized to a dihydrodiol (see D'Souza and Andersen, 1988). The studies suggest that these small fish species are useful for examining the carcinogenicity of halogenated hydrocarbons that have high environmental importance. Further studies on the metabolic activation of these compounds correlated with their carcinogenicity to these models will yield important information regarding the fate and effects of these compounds.

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7.0. Studies on the carcinogenicity and metabolism of the aromatic amine 2-acetylaminofluorene in the medaka (*Oryzias latipes*) and guppy (*Poecilia reticulata*)

Introduction

The aromatic amines are a class of chemicals that include the carcinogens benzidine and aniline as well as 2-acetylaminofluorene (2-acetamidofluorene; N-2-fluorenylacetamide; 2-AAF). Although the carcinogenicity of 2-AAF in rodents is well-known and it is widely used as a model carcinogen in initiation-promotion tests, its carcinogenicity, or the carcinogenicity of any other aromatic amine, has not been reported comprehensively tested in a fish model. Compared with DEN, MAM, and PAH's, few aromatic amines, a class of chemicals which also includes the carcinogens 2-acetylaminofluroene (AAF), benzidine, and aniline, have been widely tested for carcinogenicity in small fish species. Aromatic amines appear to be weakly to moderately carcinogenic to small fishes. Aminoazotoluene and AAF induced liver neoplasms in medaka (Hatanaka et al., 1982) and guppies (Sato et al., 1973). AAF has been used as a model carcinogen to study mechanisms of initiation and promotion in rodents. For 2-AAF to be carcinogenic, it must be N-hydroxylated by a cytochrome P-450-dependent, microsomal-bound enzyme (Weisburger, 1990). Ring hydroxylation by another P-450 enzyme appears to be a detoxification step. Similarly, these processes are not well understood in fish models although recent studies have shown that rainbow trout microsomes are capable of N-hydroxylating aniline and 4-chloroaniline (Dady et al., 1991). In this study we examined the metabolism of AAF in medaka and guppy, and found that the guppy was able to produce more of the carcinogenic metabolite of AAF than was the medaka. Correspondingly, in parallel carcinogenicity studies of AAF in the two species, the guppy developed hepatic neoplastic lesions sooner and in higher incidences than did the medaka.

Materials and Methods

Exposure methodology. For the carcinogenicity studies, medaka and guppy were exposed to AAF by two mechanisms: (1) a static single pulse exposure or multiple intermittent pulse exposures; and, (2) under prolonged static-renewal conditions. All exposures were performed in the dark or subdued light to avoid photodegradation of AAF.

Treatment groups were as follows:

(1) Pulse exposure groups

a. 1 x 6 hours
b. 1 x 12 hours
c. 2 x 12 hours, one week intervals
d. 3 x 12 hours, one week intervals
e. 4 x 12 hours, one week intervals

(2) Static renewal exposures

One group was exposed continuously for 168 hours (7 days) with renewal of AAF and control solutions every 24 hours.

Appropriate controls were included for each treatment group. Pulse exposures were conducted in one control aquarium and one AAF treatment aquarium. Medaka and guppies were exposed simultaneously in the same treatment aquarium with specimens for each treatment contained in individual mesh chambers. For each weekly pulse exposure, AAF was added to well water to produce a 10 mg/l nominal concentration, the mixture was stirred in the dark at room temperature for 4 days, filtered through a 0.2 μm Nuclepore membrane filter, and the resulting suspension diluted 1:1 with well water and transferred to the exposure aquarium. Mesh chambers containing fish were added and a sample was taken at time zero (T-0) for analysis. The control aquarium contained unamended well (diluent) water. Water samples were also taken at 6 hours (T-6) and 12 hours (T-12) when individual treatments were terminated. Each treatment consisted of 300 specimens of 6-day post-hatch medaka (wet and dry weights 3.47 and 0.57 mg/fry, respectively) and less than or equal to 48-hour post-parturition guppies (wet and dry weights means of groups 8.91 ± 0.63 and 1.86 ± 0.19 mg/fry, respectively). One large initial pool of medaka fry was used for all treatments whereas each guppy group came from a set of fry collected weekly. After the appropriate length and number of exposures, all fish were rinsed three times in well water, and the fish counted and placed in grow-out aquaria. In the static renewal test, both species were exposed simultaneously with guppies and medaka sequestered in mesh chambers in individual 4 L beakers. The AAF stock solution was prepared as described for the pulse exposure. Four 4 L beakers were used, 1 for control and 1 for AAF for each of the two species. Total volume in each beaker was 3 L with daily replacement being accomplished with a single 4 L AAF/well water preparation. A time zero-hour water sample was taken immediately after the mesh chamber containing fish was introduced into the exposure aquarium. The 24-hour sample was taken the following day before the mesh chamber was removed to a new 4 L beaker containing fresh solution (well water or AAF). Then, another time zero hour sample was taken. At the end of the 7 days exposure, the fish were rinsed, counted and placed in grow-out aquaria. Concentrations in both types of exposures were around 1.0 mg/l.

Determination of AAF concentrations. Because there was no published methodology for the measurement of AAF in water, some preliminary studies were necessary. The aromatic ring structure of AAF suggested that AAF could be measured by fluorescence spectrophotometry which would permit direct analysis without prior extraction. However, the intensity of fluorescence of AAF in water solution was insufficient for the sensitivity required in this procedure. Efficiency of extraction from a water medium using various solvents having slightly different polarities was then determined. Hexane, benzene, and dichloromethane were used to extract saturated solutions of AAF from distilled water. Dicholoromethane was determined to be the most efficient solvent for extraction. Gas chromatographic analysis using a flame ionization detector was employed and various column conditions were tested to find the best conditions for measurement of AAF. Methodology was finalized and monitoring was performed using a Perkin-Elmer 3920 gas chromatograph 15 m x 0.25 mm (i.d.) fussed silica glass capillary column with 0.25 μm coating of DB-1 (J&W Scientific). Quantitation was achieved by internal standard method using a Perkin-Elmer Sigma 10 Data System. Histopathological evaluations followed protocols described above.

Metabolic studies with the medaka. Hepatic metabolic studies with 2-AAF were conducted on medaka. Medaka were at least three months old at the time of exposure. An acute, static exposure of AAF to medaka was conducted at the Gulf Coast Research Laboratory. Six 4-L flasks containing a nominal concentration of 10 ppm AAF in well water was stirred in the dark for four days. Each was then filtered through a 0.2 μm Nucleopore filter and all filtrates added to an equal volume of well water in the exposure aquarium. The control aquarium contained unamended well water. Immediately prior to addition of the fish, duplicate water samples were collected for quantitation of AAF concentration. Aquaria were held in the dark throughout the 48- to 72- hour exposure period. Test and control fish were periodically examined under subdued light, and fish were not fed during the exposure. AAF concentration measured 2.07 mg/L. AAF was diluted to about 1.0 mg/L and AAF-exposed fish were transported in the diluted AAF to the C.V. Whitney Laboratory, St. Augustine, Florida, for the biochemical analyses.

For the metabolic studies, livers were removed, weighed, and homogenized in ice cold 1.15% KCl, 0.02M HEPES buffer (pH 7.4) adjusted with the protease inhibitor phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 13,000 x g at 0 °C for 20 minutes, the pellet discarded, and the resulting supernatant centrifuged at 176,000 x g at 0 °C for 45 minutes to produce cytosolic and microsomal fractions. The microsomal fractions of control and AAF-exposed specimens were incubated with [9-14C]AAF according to Juchau et al. (1975) to search for metabolite profiles. Metabolites were identified by thin-layer chromatographic techniques using silica gel 150A TLC plates of 250 μm thickness with preadsorbent spotting area. Standards run simultaneously included N-hydroxy-2-AAF, 1-hydrdoxy-2-AAF, 3-hydroxy-2-AAF, 5-hydroxy-2-AAf, and 7-hydroxy-2-AAF. Another aliquot of each microsomal fraction was incubated with unlabelled N-OH-AAF and UDP-glucuronyl transferase activity was assayed using radiolabelled cofactors. The glucuride produced from this enzyme is a weakly reactive electrophile that is excreted in urine. In this way, the medaka's ability to detoxify the proximate carcinogen was assessed. Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to Laemmli (1970) using a 4% acrylamide stacking gel and a 7.5% acrylamide separating gel introducing 20 μg microsomal protein per well. Epoxide hydrolase with styrene oxide (Oesch et al., 1971) was assayed in the microsomal fraction of both control and AAF-exposed fish. Preliminary tests produced a slight induction in a protein of approximately 49 K Dalton molecular weight upon exposure to AAF. This is within the weight range of epoxide hydrolase enzymes. Therefore, the appearance of any novel protein bands in the gel was compared with epoxide hydrolase activity. Protein content was determined using the Folin and Ciocalteu's phenol reagent methods according to Lowry (1951).

Results

Histopathology. AAF appeared to cause neoplastic lesions in livers of the guppy earlier and in higher incidences than in th. medaka. The incidences of those lesions are shown in Tables 7.1 and 7.2 for the medaka and guppy, respectively. The response in the two highest exposure levels in the guppy tests were statistically significant by Fisher's exact test. The hepatic neoplastic lesions diagnosed in the guppy, however, were not considered malignant. Most of the lesions were either foci of cellular alteration (altered foci) or hepatocellular adenomas. None have been diagnosed as hepatocellular carcinomas.

Biochemistry. To investigate the hepatic bioactivation of 2-AAF in medaka, a preliminary 48-hour exposure to 8.6 ppm 2-AAF was run and the activities of a series of mixed function oxidase enzymes (MFO's) assayed. Results are summarized in Table 7.3. The activity of the MFO ethoxycoumarin O-deethylase was suppressed by 2-AAF exposure and a new protein band at approximately 49 KDaltons was observed in the electrophoretic separation of the microsomal fraction of AAF-exposed fish as compared to controls. There was no difference in the bands of the cytosolic fractions of both treatments. For the definitive study, approximately 100 adult medaka were exposed to 2.07 ppm 2-AAF for 48 hours with 100 untreated medaka serving as controls.

Table 7.1. Incidence of combined hepatocellular neoplastic lesions (altered focus, adenoma and carcinoma) in the medaka (*Oryzias latipes*) exposed to AAF.

Exposure Group	24 wk	36 wk	52 wk
Ctl/12hx4	0/62	0/75	0/89
Constant Ctl	0/69	1/77	1/108
AAF 6 hrx1	0/76	0/77	2/105
AAF 12hx1	0/74	1/80	0/68
AAF 12hx2	2/75	0/73	3/82
AAF 12hx3*	0/1	1/32	0/0
AAF 12hx4	0/66	0/75	3/88
AAF Constant	3/77	0/77	3/84

*High mortality of unknown cause

Table 7.2. Incidence of combined hepatocellular neoplastic lesions (altered focus, adenoma and carcinoma) in the guppy (*Poecilia reticulata*) exposed to AAF.

Exposure Group	24 wk	36 wk	52 wk
Ctl/12hx4	0/90	1/70	0/92
Constant Ctl	0/84	0/69	1/82
AAF 6 hrx1	0/97	1/74	5/85
AAF 12hx1	0/94	0/73	5/106
AAF 12hx2	2/89	1/76	4/95
AAF 12hx3	4/92	3/72	8/68
AAF 12hx4	3/99	7/72	3/68
AAF Constant	3/86	7/75*	8/98*

*Statistically significant at $p \leq 0.05$ when compared by Fisher's exact test with the incidence in the Constant Control group.

Table 7.3. Biotransformation pathways in control and AAF-treated medaka.

Parameter Measured	Control	Treated
Protein Yield, mg/g liver		
Microsomes	9.64	15.4
Cytosol	52.00	44.4
Oxygenation of AAF Total, pmole/min/mg protein	256	68.5
7-OH	210	48.4
5-OH	20	8.8
3-OH	4	1.9
1-OH	2	1.3
N-OH	5	0.7
Glucuronyl transferase activity, pmole/min/mg protein		
4-Methyl umbelliferone	556	680
3-Hydroxy AAF	181.2	244.6
Epoxide hydrolase, nmole/min/mg protein	1.51	1.11
GSH-S-transferase, nmole/min/mg protein	1370	1814
Sulfotransferase, pmole/min/mg protein		
4-Methyl umbelliferone	129.6	40
3-Hydroxy AAF	25.2	30.0
N-Hydroxy AAF	184	75

Exposed medaka were transferred to a dilute (about half strength) solution of 2-AAF for 24 hours during which time they were transported to the Whitney Laboratory where they were sacrificed. Incubation of 2-AAF with the microsomal fractions of both treatments resulted in a combined four-fold increase in the amount of combined 2-AAF metabolites in the control treatment compared with that in the 2-AAF pretreated microsomes. The major AAF metabolite formed *in vitro* was 7-OH-AAF, followed by 5-OH-AAF, both of which indicate ring hydroxylation. The carcinogenic intermediate, N-OH-AAF, was also produced demonstrating, at least qualitatively, the activation capability for aromatic amines of medaka hepatic enzyme systems. In summary, 2-AAF depresses hepatic microsomal oxidative enzyme activities whereas it increases glutathione S-transferase activity. Exposure to 2-AAF does not seem to affect the activities of epoxide hydrolase or of glucuronyl transferase. Medaka unexposed to 2-AAF appear to be capable of hydroxylating AAF *in vitro* mainly to ring metabolites and to a lesser extent to the N-metabolite, N-hydroxy-AAF, the proximate carcinogen.

The metabolism of acetylaminofluorene (AAF) in guppies was examined to compare it with that in the medaka and relate metabolic capability to the carcinogenicity of AAF to both species. The results for the positional metabolism of AAF in the guppy is summarized in Table 7.4.

Table 7.4. Positional metabolism of AAF in microsomes from untreated and AAF-treated guppies (*Poecilia reticulata*).

	pmole/min/mg protein	
Metabolite	Treated	Control
7-HydroxyAAF	102.5 ± 21.9	427.7 ± 23.6
5-HydroxyAAF	8.69 ± 1.64	63.41 ± 4.90
Unknown #1	0.78 ± 0.39	2.91 ± 0.54
Unknown #2	1.56 ± 0.35	5.88 ± 1.43
3-HydroxyAAF	1.17 ± 0.19	2.82 ± 0.72
1-HydroxyAAF	0.55 ± 0.17	1.44 ± 0.05
N-HydroxyAAF	0.31 ± 0.18	1.01 ± 0.12
Total metabolized	126.8 ± 15.43	521.8 ± 26.4

The data show that the major metabolite is the 7-hydroxy metabolite as it was in the medaka, but there was very little N-hydroxy formed in either treated or control fish. If, as we suspect, there were residues of 7-hyroxy-AAF and AAF in the microsomes from the treated animals, this may well be the reason for the apparently low monooxygenase activity in the treated fish. The presence of 7-hydroxy AAF could also explain the lower glucuronosyltransferase activity with 7-hydroxy AAF in the AAF-treated microsomes, due to dilution of the substrate with material from the exposure. In retrospect, it would probably have been better to allow the specimens to depurate overnight before they were analyzed. Overnight depuration would probably have resulted in increased values for pmole product formed/min/mg/protein, and although it probably would not have altered the pattern of having 7-hydroxy as the major metabolite we perhaps could have seen whether the rate of formation of N-hydroxy was elevated. It is difficult to explain the carcinogenic sensitivity of guppies to AAF based on these results, although it may be significant that the total metabolism of AAF was double in the control guppies (0.522 nmole/min/mg protein), relative to control medaka (0.256).

Discussion

To our knowledge, studies on the hepatic metabolism of AAF in the medaka represent the first time that the relative refractoriness of a fish species to the carcinogenic effects of AAF has been related to the fish's inability to metabolize adequately the compound to is proximate carcinogen by N-hydroxylation relative to its ability to detoxify the compound by ring hydroxylation. Furthermore, only one other study, that of Dady et al. (1991), has demonstrated N-hydroxylation of an aromatic amine in any fish species.

Two previous studies have suggested that aromatic amines, particularly AAF, can be carcinogenic to fish species. Sato et al. (1973) exposed guppies beginning at one month of age to 30 mg AAF/100 g powdered diet for "several months." Hepatic nodules, probably either altered foci or adenomas, were observed in 1 of 3 male guppies examined at 11 months and in 3 of 7 females examined at 13 months. Pliss and Khudoley (1975) induced hepatic tumors in guppies by dietary exposures to o-aminoazotoluene and 4-dimethylaminoazobenzene. Hatanaka et al. (1982) also observed hepatic neoplasms in medaka following a 12-week dietary to 600 ppm o-aminoazotoluene. However, feeding studies of 2-AAF (300 or 150 ppm in food administered for 12 weeks) did not induce hepatic neoplasms in medaka examined up to 36 weeks post-initial exposure (Hatanaka et al., 1982). In the present study, AAF caused a statistically significant increase in combined hepatic neoplastic lesions in the guppy. The low carcinogenic potency of this compound, however, was evidenced by the fact that many of the induced lesions, in spite of the fact that they persisted for 6 and 9 months post exposure, did not appear robust or actively progressing to more aggressive lesions. This is the first time in our studies that we have observed the apparent regression of carcinogen-induced lesions, even ones such as those we designate as altered foci which are generally terminal lesions in rodents. This poses some interesting questions regarding the nature of neoplastic initiation in small fish models. Perhaps the persistence of neoplastic lesions is related to the dose x time or to the strength of the test carcinogen. It would also be interesting to compare the carcinogenic effects of the proximate carcinogen, N-OH-AAF, on both species.

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8.0. Studies on the effects of cadmium on medaka and guppy

Introduction

Cadmium is widely distributed in nature and affects man through occupational exposures such as in smelters, through food consumption mainly in the form of contaminated seafood, and through tobacco use (Kazantzis, 1987). Absorbed cadmium is eventually bound to a low molecular weight metal binding protein, metallothionein. Metallothionein-bound cadmium accumulates mainly in the kidney proximal tubular cells. Cadmium is associated with unusual patterns of carcinogenesis. Following subcutaneous injection in rats, it induces injection-site sarcomas (mainly fibrosarcomas and rhabdomyosarcomas) as well as reproductive toxicity and neoplasms in males (Haddow et al., 1961; Kazantzis and Hanbury, 1966; Lucis et al., 1972). Cadmium administered in drinking water, in the diet, or by gavage, however, was not determined to be carcinogenic in rats (Loser, 1980).

We conducted cadmium exposures with medaka in which the fish were exposed for various periods of time to waterborne cadmium at near toxic levels. Our rationale was that this type of exposure would combine skin contact exposure, epithelial (gill) uptake exposure, and possible enteric exposure through consumption of the cadmium-containing water. Although cadmium exposure was not associated with increased carcinogenesis in medaka in this bioassay and there are gaps in the study, the study is presented here in detail to compare these negative results with studies involving other metals, fish species, and methods of exposure. The study was repeated using intraperitoneal injection as the route of exposure.

Materials and Methods

Waterborne exposure. Three hundred 6 to 7 day old medaka (mean wet and dry weights 2.60 and 0.48 mg/fry, respectively, based on a random sample of fifty 6 day old fry) were assigned to each of the following treatment groups according to procedures described above in section *3.6. Randomization and sampling*:

1. Nominal 30 ppb Cd, 1 x 6 hours
2. Nominal 30 ppb Cd, 1 x 12 hours
3. Nominal 30 ppb Cd, 1 x 24 hours
4. Nominal 30 ppb Cd, 2 x 24 hours, one week intervals
5. Nominal 30 ppb Cd, 3 x 24 hours, one week intervals
6. Nominal 30 ppb Cd, 4 x 24 hours, one week intervals

Each of these groups were matched with control groups which were handled identically to their cadmium-exposed counterparts. Preliminary experiments revealed that cadmium chloride prepared in distilled water and added to well water normally used in our freshwater bioassay studies precipitated, presumably due to a combination of high alkalinity (240 mg/l calcium carbonate) and low hardness (1.8 mg/l calcium carbonate) in our well water. Therefore, we used a "synthetic" water that contained 96.0 mg $NaHCO_3$, 60.0 mg

CaSO4.2H2O, 60 mg MgSO4, and 4.0 mg KCl per liter of distilled water. Following the last exposure, specimens were removed to grow-out aquaria until sampled for histological examination as described above in Section *3.0. Methodology.*

Results and Discussion

Histopathological studies did not reveal a carcinogenic response in cadmium exposure medaka (Table 8.1).

Table 8.1. Incidence of combined hepatocellular neoplastic lesions (altered focus, adenoma and carcinoma) in the medaka (*Oryzias latipes*) exposed to waterborne cadmium.

Exposure Group	24 wk	36 wk	54 wk
Control 24 hr x 4	0/48	1/34	*
Cd++ 24 hr x 3	NE	NE	0/25
Cd++ 24 hr x 4	0/75	0/58	*

NE= Not Examined
*No specimens remaining in test

Although we paid special attention to the possible development of hepatocellular neoplastic lesions, neoplastic lesions did not appear to develop in any other organs either. The absence of any kind of neoplastic response concerned us and we can apply the same analysis to this study that we did with the 1,1,2,2-tetrachloroethane (TeCE) study described above in section 4.0 which also yielded negative results. In contrast with TeCE carcinogenesis in mammals which is highly species specific, there appears to be little species-related sensitivity related to cadmium carcinogenesis in mammalian models. Given that there is little understanding of the carcinogenic mechanism of any metal including cadmium (Furst, 1987), it seems unlikely that the medaka would not be sensitive because of some species-related factor. Furthermore, because exposure concentrations were near the toxic levels to the medaka, it is unlikely that a exposure concentrations were inadequate. Before we were prepared to assert that cadmium is not carcinogenic to small fish, we repeated the tests using multiple intraperitoneal injections to deliver cadmium chloride to adult specimens of both the medaka and guppy.

Intraperitoneal injection studies. Because the study in which medaka were originally exposed to water-borne cadmium was compromised possibly by interactions between cadmium and the exposure water which probably interfered with the compound being incorporated into the organism, medaka and guppy were exposed to cadmium by intraperitoneal injection. Preliminary histopathological examination revealing renal cytotoxicity indicates that the specimens received the compound internally since cadmium is well-known to induce renal damage in fishes as well as in mammals. These injection studies were accompanied by severe mycobacterial granuloma reactions in both species. Histologic examinations (Tables 8.2 and 8.3) of specimens show that cadmium exposure did not initiate carcinogenesis and we must assume that cadmium is not carcinogenic to medaka or guppies under the conditions used in these studies.

Table 8.2. Incidence of combined neoplasms in the medaka (*Oryzias latipes*) exposed to Cd++ by intraperitoneal injection.

Exposure Group	13 wk	24 wk
Cd++ x 1	0/34	0/34
Cd++ x 1/Ctl	0/15	0/19
Cd++ x 2	0/30	0/44
Cd++ x 2/Ctl	0/14	0/67
Cd++ x 3	0/25	0/20
Cd++ x 3/Ctl	0/16	0/38
Cd++ x 4	0/18	0/19
Cd++ x 4/Ctl	0/16	0/25

Table 8.3. Incidence of combined neoplasms in the guppy (*Poecilia reticulata*) exposed to Cd++ by intraperitoneal injection.

Exposure Group	13 wk	24 wk	36 wk	52 wk
Cd++ x 1	0/8	0/2	0/1	0/15
Cd++ x 1/Ctl	0/23	0/1	NE	*
Cd++ x 2	0/29	NE	NE	*
Cd++ x 2/Ctl	0/2	0/3	NE	0/7
Cd++ x 3	0/8	0/65	NE	*
Cd++ x 3/Ctl	0/4	0/1	NE	*
Cd++ x 4	0/11	0/27	0/46	*
Cd++ x 4/Ctl	0/12	0/26	2/77	*

NE=Not examined
*No specimens remaining in test

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9.0. Studies on the effects of acrylonitrile on medaka and guppy

Introduction

Acrylonitrile is a bulk industrial chemical used primarily in the synthesis of acrylic and modacrylyic fibers for clothing and home furnishing and for the production of various resins. Acrylonitrile is also used as an absorbent, an anti-stall additive to gasoline, a fumigant for stored tobacco, for flour milling and bakery food processing equipment, and in pesticides. As an industrial chemical, acrylonitrile is widely produced, transported, and utilized in many countries worldwide. According to IARC (1979), acrylonitrile not only occurs in work places but also in the air near industrial production and processing sites, in rivers when it has been discharged in effluent from chemical and latex manufacturing plants and thus as trace amounts in drinking water. Acrylonitrile has also been found as a contaminant of fish and shellfish, and in food treated with acrylonitrile-containing fumigants. Acrylonitrile also occurs in food containers and packaging materials and there is concern for it contaminating the stored materials.

Epidemiological studies of workers exposed to acrylonitrile showed excesses in colon and lung cancers (Finklea, 1977). Results of toxicological and metabolic studies, however, suggest that acrylonitrile does not fit the criteria of a typical carcinogen. It is mutagenic in some assays (Rabello-Gay and Ahmed, 1908; Milvy and Wolff, 1977) and appears to bind covalently with nucleic acids (see Farooqui and Ahmed, 1983). In rats, acute exposure to acrylonitrile has been shown to cause adrenocortical necrosis (Szabo et al., 1982) but not hepatotoxicity (Silver et al., 1982). Several bioassays to determine the carcinogenicity of acrylonitrile have been conducted on rodents. Maltoni et al. (1977) exposed Sprague-Dawley rats to acrylonitrile by inhalation (up to 60 ppm, 5 days weekly, 52 weeks) and detected an increase in different types of tumors, most noticeably brain gliomas. In a parallel study, in which acrylonitrile was administered by stomach tube (olive oil carrier, 5 mg/kg, once daily, 3 times weekly, 52 weeks) no carcinogenic effects were detected. Gallagher et al. (1988) exposed male Sprague-Dawley rats to up to 500 ppm acrylonitrile in drinking water for two years and observed that tumors of Zymbal's gland occurred in a dose-related fashion. There was a trend toward the development of forestomach papillomas noted in rats receiving the highest concentration of acrylonitrile. Fischer 344 rats administered acrylonitrile in the drinking water for 12-18 months developed primary brain tumors that were difficult to classify (Bigner et al., 1986). In this project, we conducted studies to determine whether waterborne acrylonitrile induced neoplastic lesions in medaka and guppies.

Materials and methods

Acrylonitrile (AN) was investigated in multiple pulse tests on the guppy and medaka. Specimens were exposed to a single nominal concentration (about 35 ppm) which was determined to be minimally toxic in preliminary range-finding tests. Specimens were assigned to one of six test groups according to procedures described in section *3.6. Randomization and sampling*. The test groups included three treatment groups and three control groups. Treatment groups were administered AN once (1X), twice (2X) or four times (4X). Each exposure lasted for 24 hours after which the specimens were removed to clean water. Each of the multiple exposures (2X and 4X) was conducted at weekly intervals. Each control group (1X, 2X, and 4X) was handled in the same way as a corresponding exposure group with the exception of being exposed to AN.

Following each pulse exposure, each group of fish was removed from the test solution, rinsed, and counted. A sample of approximately 20 fish from each group receiving its final pulse exposure was removed for histological evaluation. All remaining fish were transferred to either holding or growout aquaria to await sampling.

Results and Discussion

Tables 9.1 and 9.2 show the results of waterborne exposures of medaka and guppies to acrylonitrile.

Table 9.1. Incidence of hepatocellular neoplastic lesions (altered focus, adenoma, carcinoma) in medaka (*Oryzias latipes*) exposed to waterborne acrylonitrile.

Exposure Group	13 wk	24 wk
x 4 Control	0/4	0/71
x 1 AN	0/18	NE
x 2 AN	0/14	NE
x 4 AN	0/51	0/37

Table 9.2. Incidence of hepatocellular neoplastic lesions (altered focus, adenoma, carcinoma) in the guppy (*Poecilia reticulata*) exposed to waterborne acrylonitrile.

Exposure Group	13 wk	24 wk
x 1 Control	0/6	0/68
x 1 AN	0/9	NE
x 2 AN	0/19	NE
x 4 AN	0/14	0/70

Several histologic features suggest toxic and, possibly, proliferative effects of AN on the guppy. These included the presence of mitotic figures in hepatocytes which is uncommon in control specimens. Also, single cell necrosis occurred in the liver of at least one AN-exposed specimen. A third condition was one that we commonly see associated with carcinogen exposure and that was a heterogenous or mottled appearance of the liver with regard to the presence of fat and glycogen in the hepatocytes. This occurred in several AN-exposed guppies. At the present time, however, we conclude that AN does not show evidence of carcinogenicity under the exposure and evaluation conditions of the present study.

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10.0. Studies on the effects of methapyrilene in the medaka and guppy

Introduction

Methapyrilene is an antihistaminic drug that was widely used as an over-the-counter sleep aid before it was shown to be hepatocarcinogenic to rats (Connors, 1984). In rodent carcinogenicity studies, methapyrilene incorporated into the diet and administered over 64 weeks, induced hepatic neoplasms (hepatocellular carcinomas and cholangiocarcinomas) in nearly 100% of the exposed rats and almost 50% of them developed metastatic lesions (Lijinski et al., 1980). Methapyrilene represents a class of carcinogens that possesses methylamino and dimethylamino groups that can be metabolized to carcinogenic intermediates. That class incudes both carcinogenic and noncarcinogenic analgesics, some antipsychotic drugs, diazepam, and methylene blue (Connors, 1984).

Methapyrilene is no longer manufactured. Dr. W. Lijinsky (BRI-Basic Research Program, Chemical Carcinogenesis Program, NCI-Frederick Cancer Research Facility, Frederick, Maryland, graciously supplied us with the compound for our tests.

Materials and methods

Medaka and guppies were exposed to a single nominal concentration (about 2 mg/L methapyrilene), determined not to be lethally toxic in preliminary range-finding tests. Specimens were assigned to one of six groups, three treatment and three control following procedures described above in section *3.6. Randomization and sampling*. One treatment group was administered methapyrilene (MP) once (X 1), twice (X 2), and or four times (X 4). Each exposure was planned to be for 24 hours. In the multiple (X 2 and X 4 groups), MP exposures were administered once a week. Each exposure group was matched by a control group (X 1, X 2, and X 4) each of which was handled similarly to its exposure counterpart except for the MP exposure.

Test specimens were reared according to procedures outlined above in section *3.0. Methodology*. Following each exposure, each group of fish was removed from the test solution, rinsed, and counted. A sample of approximately 20 fish from each group receiving its final exposure was removed for histological evaluation. All remaining fish were transferred to either holding or growout aquaria.

In the guppy test, the first exposure was terminated after only 6 hours exposure because behavioral responses exhibited by the specimens such as loss of equilibrium and erratic rapid movements, made it unlikely that the fish could survive an overnight exposure. We then decided to conduct subsequent methapyrilene exposures at a nominal 1.0 ppm, rather than 2.0 ppm.

In the first exposure of the medaka test, specimens began dying about 2 hours after being placed in the treatment beakers. To avoid any additional mortality, the exposure was prematurely terminated at that time. The decision was then made to conduct subsequent methapyrilene pulses at a nominal 1.0 ppm, rather than 2.0 ppm.

Following exposures, specimens of both species were removed to grow-out aquaria for later sampling as described above in section *3.0. Methodology*.

Results and Discussion

Histopathological results for the medaka test are summarized in Table 10.1. Fourteen whole fish specimens from the x 1 control group were examined at 1 day, 4 from the x 1 MP group at 1 day post exposure, 13 from the x 2 MP group at 1 week post exposure, and 17 from the x 4 MP group at 3 weeks post exposure. Several specimens from the x 2 MP (1 week post exposure) showed severe gill necrosis which might account for some of the unexpected toxicity of this compound. Examination of 70 specimens from the high exposure (x 4) group at 24 weeks post exposure revealed no hepatocellular neoplastic lesions (Table 10.1), or any other type neoplasm. We must conclude, then, that MP showed no evidence of carcinogenicity in the medaka, at least under the conditions of this test.

Table 10.1. Incidence of hepatocellular neoplastic lesions (altered focus, adenoma, carcinoma) in medaka (*Oryzias latipes*) exposed to waterborne methapryrilene (MP).

Exposure Group	13 wk	24 wk
x 4 Control	0/8	0/70
x 1 MP	0/2	NE*
x 2 MP	0/13	NE
x 4 MP	0/17	0/70

*NE= Not Examined

For the guppy, 13 whole specimens were examined histologically from the x 1 control group at 1 day, 18 from the x 1 MP group at 1 day post exposure, 18 from the x 2 MP group at 1 week post exposure, and 17 from the x 4 MP group at 3 weeks post exposure. Lesions in MP-exposed specimens suggested toxic effects. Several specimens in the x 1 MP, x 2 MP, and x 4 MP group had evidence of liver degeneration. Scattered throughout the livers of specimens from the x 4 MP group were "signet ring" nuclei that suggested cytotoxicity. Histopathological examination of 70 specimens from the high (x 4) exposure group did not reveal any hepatocellular neoplastic lesions (Table 10.2), or any other type of neoplasm. Under the conditions of this test, methapyrilene did not show evidence of carcinogenicity to the guppy. The early hepatotoxic effects, especially in the guppy, however, suggest that hepatocarcinogenesis may develop at some later time period.

Table 10.2. Incidence of hepatocellular neoplastic lesions (altered focus, adenoma, carcinoma) in the guppy (*Poecilia reticulata*) exposed to waterborne methapyrilene (MP).

Exposure Group	13 wk	24 wk
x 1 Control	0/7	0/43
x 1 MP	0/9	NE*
x 2 MP	0/18	NE
x 4 MP	0/17	0/70

*NE= Not Examined

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11.0. Studies on the effects of dibenzocarbazole on the medaka

Introduction

7H-Dibenzo(c,g)carbazole (DBC), an N-heterocyclic compound, is an environmental pollutant that is associated with combustion-related processes such as cigarette smoke, soots and tars, wood smoke, diesel exhaust, and synthetic fuel material. DBC is a potent carcinogen in mouse tissues and, compared with other carcinogenic polynuclear aromatic hydrocarbons (PAH) such as benzo(a)pyrene (BaP), is usually hepatocarcinogenic in mice (Schurdak and Randerath, 1989). A variety of N-heterocyclic compounds have been found in industrially polluted sediments in the Puget Sound, Washington, area and have been implicated in hepatocarcinogenesis in English sole associated with the sediments (Malins et al., 1982). Whereas N-heterocyclic analogues of PAH occur in concentrations 1 to 3 orders of magnitude less than that of BaP, their apparently high carcinogenic potency coupled with their expected increase in the environment make them important compounds for study (Santodonato et al., 1981; IARC, 1972). In this study, we conducted a carcinogenesis bioassay with DBC on the medaka. Although the study failed to demonstrated DBC carcinogenicity on the fish model, we established methodological approaches to examine this kind of poorly soluble environmental polycyclic aromatic compound in small fish carcinogenesis systems.

Materials and methods

Analytical determinations. Because we were unaware of any studies in which DBC had been tested in waterborne exposures, preliminary studies had to be directed toward understanding the properties of DBC in water. Those studies are summarized here.

Because of the known intense fluorescence of carbazole, we thought that DBC might possess similar fluorescent properties on which we could base a technique for analytical detection. Anticipated advantages of using fluorescence as a measuring technique over other techniques would be speed and simplicity of analyses and consequently reduced analytical variability. A high level of sensitivity would also be desirable since it could reasonably be expected that measurements in exposure media at parts per billion concentrations level might be necessary.

First, we prepared a primary standard by weighing out 1.07 mg of DBC and dissolving it in 10 ml of CH_2Cl_2 yielding a 107 ppm DBC solution. Serial dilutions were made that resulted in standards that ranged from 10.7 ppm to 0.107 ppb. A total excitation emission scan on a Perkin Elmer MPF-44 fluorescence spectrofluorometer gave the principal excitation λ at 366.5 nm with a secondary excitation λ of 351.0 nm. In the emission mode with excitation set at 366.5 nm the principal emission λ's were 370.2 nm followed by 389.7 nm. Having the principal excitation and emission wavelengths displaced by only 4 nm was unusual. Confirmation of the principal emission λ was achieved by scanning the emission with an excitation λ of 351.0 nm. A "false" emission peak always appeared in the emission spectrum at the excitation λ, therefore an emission spectrum run under maximum sensitivity conditions (i.e. excitation λ set at 366.5 nm) would have the maximum emission peak (370.2 nm) preceded by one at 366.5nm which is not sufficient separation for exacting analysis at trace levels. We then proceeded to use the secondary excitation λ of 351.0 nm and make analytical measurements based on fluorescence intensity at 370.2 nm emission λ.

Emission scans of standards down to and including 0.107 ppb DBC using the 351.0 nm excitation λ gave almost no fluorescence interference from the solvent and a sensitivity (concentration in CH_2Cl_2 yielding a signal 3 x that of background noise) of 0.2 ppb. Furthermore, analyses of extracts of well water yielded spectra with no perceptible fluorescence at 370.2 nm, which is a distinct analytical advantage for sample analysis of well water preparations of the test compound.

<u>Solubility</u>. Because the exposures were to be waterborne, it was important to determine the solubility of DBC. Typically in aquatic toxicological experiments, excessive amounts of poorly soluble test compounds are introduced to stock tanks presuming that after extended periods of stirring, solutions would reach saturation and excess material could then be discarded. Alternatively in toxicology tests, test material has been introduced as a solution in a solvent carrier often in amounts exceeding solubility resulting in precipitation of the test compound. Two problems arise from both of those techniques. First, these approaches can be justified only for compounds of low cost and ready availability. Second, studies have shown that media prepared in these ways often are not true solutions but actually are combinations of solute and microfine particulate test compound. These particulates can vary in both size distribution and quantity so that test media are not readily reproduced from test to test even in the same laboratory.

Because DBC is not commercially available, we acquired the compound from Dr. David Warshawsky, University of Cincinnati Medical Center, who synthesizes the compound for continuing research in his laboratory. For our study, he synthesized and provided 10 mg DBC for our initial analytical studies. Because of the value of this compound, determining the actual solubility of DBC could then allow us to prepare the exact required quantities of DBC thereby substantially reducing waste.

The solubility of DBC was determined by a technique developed by T. Lytle and S. Manning which is described as follows. A 1 L beaker was outfitted with a 50 ml beaker cemented to the center of the bottom. We coated 1.10 mg of DBC onto 25 cm^2 of etched glass beads by slowly pouring a solution in CH_2Cl_2 of the DBC over the beads which were gently stirred while being bathed in a vigorous stream of nitrogen. This helped assure that the beads were evenly coated and that material did not coat the beaker. After additional air drying, the beads were carefully placed in the ring outside the 50 ml beaker. Then, 500 ml of well water was carefully poured into the central beaker overflowing into the larger beaker. The clarity of the solution indicated that material had not flaked off the beads. A stirring bar was introduced to the central beaker which was then placed on a stirring plate. The entire apparatus was covered in foil to shield it from light. The stirring rate was sufficient to swirl the water without causing mechanical agitation of the beads. Triplicate samples were withdrawn at 0.5, 1, 2, 4, 8 and 24 hr intervals, extracted with CH_2Cl_2 and concentration measured by comparison of fluorescence intensity measured against standards prepared in CH_2Cl_2 and back extracted with well water to simulate sample processing.

Excitation λ of 351.0 nm was maintained with emission scan from 348 nm to 425 nm to examine samples for the characteristic spectrum of DBC and presence of artifacts. Concentration measurements were based upon peak heights at the 370.2 nm emission λ. The theory of operation was that when concentrations plateaued, DBC was at its saturation solubility. Assuming no loss of DBC in transfers or adsorption to glass, 1.10 mg DBC was sufficient to produce a 2.2 ppm solution of DBC. This amount was considered to be at least 2 orders of magnitude more than the expected solubility of DBC in water under natural circumstances. The solubility of DBC was high suggesting that even at 1 ppm the solubilizing chamber may have depleted all available DBC before saturation was reached (Table 11.1). The experiment was repeated with 1.77 mg of DBC and sample withdrawals at only 4-hour and 24-hour intervals. Concentrations after 4 hours (the time at which the earlier experiment plateaued) were higher in this experiment and may still not have reached maximum solubility. However, we decided that this concentration exceeded levels that were anticipated in exposure media and no further efforts to reach "true" saturation were necessary. Table 11.1 shows that the solubility of DBC in GCRL well water is around 1 ppm.

Table 11.1. Solubility of Dibenzocarbazole in Well Water[1]

Amt. DBC	Concentration in µg/l (ppb) ±SD					
	½ hr	1 hr	2 hr	4 hr	8 hr	24 hr
1.10 mg	426±12.7	600±13.7	869±26.1	990±14.4	977±23.1	735±57.0
1.77 mg				1130±44.4		464±20.0

[1]Concentration of DBC in well water measured in triplicate on samples withdrawn from tank in which DBC-coated glass beads were continuously stirred.

Other chemical/physical properties of DBC. Two unusual phenomena were noted during preparation of these solutions. One batch of CH_2Cl_2 used in the laboratory contained degradation residues of HCl and preparations of standards in this solvent possessed no fluorescence. The nitrogen group of the DBC apparently can become protonated with subsequent complete loss of DBC fluorescence. If DBC behaves like other compounds such as ammonia, cyanide, and hydrogen sulfide that can exist in undissociated and free form with the free form exhibiting greater biological activity, then the chemical speciation of DBC in solution is an important consideration. Future studies should be directed toward determining the distribution of DBC and $DBCH^+$ to assure that the pH of exposure media is sufficiently high to maintain DBC in predominantly the free form.

During the solubility determinations, we also observed that the level of DBC had declined markedly by the 24-hour sampling period. This may have been due to several factors alone or together. Adsorption to glass surfaces may have depleted the stock of DBC that was going into solution with a resulting decline in the later samplings or there may have been degradation under the conditions of this preparation. In either event, the behavior of DBC and similar compounds must be factored into the design of the exposure media in the actual testing.

Carcinogenicity tests. For the carcinogenicity tests, medaka were exposed to a single concentration of dibenzocarbazole (DBC) (about 1 mg/L) which prepared as described above and considered to be at the solubility limit of the compound. Specimens were assigned to one of six groups, three treatment and three control following procedures described in section *3.6. Randomization and sampling*. One treatment group was exposed once (1 X), another treatment group was exposed twice (2 X), and a third treatment group was exposed four times (4 X). Control groups (1 X, 2 X, and 4 X) parallel exposure groups and were treated similarly except for DBC exposure. Each exposure was scheduled to be 24 hours in duration. A one-week delay intervened between multiple pulses. Following each exposure, each group of fish was removed from the test solution, rinsed, and counted. A sample of approximately 20 fish from each group receiving its final pulse exposure was removed for histological evaluation. All remaining fish were transferred to either holding or growout aquaria.

Results and Discussion

Results of histopathologic examinations are shown in Table 11.2. At this point it appears that this compound is not carcinogenic to the medaka under the conditions it was tested.

Table 11.2. Incidence of hepatocellular neoplastic lesions (altered focus, adenoma and carcinoma) in the medaka (*Oryzias latipes*) exposed to dibenzocarbazole (DBC).

Exposure Group	24 wk	36 wk	52 wk
x1 Control	0/45	0/69	0/35
x1 DBC	0/15	NE	NE
x2 DBC	0/11	NE	NE
x4 Control	0/12	NE	NE
x4 DBC	0/49	1/50	1/30

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12.0. Studies on the effects of 4-aminobiphenyl on the medaka and guppy

Introduction

4-Aminobiphenyl (ABP; 4-biphenylamine) is an aromatic amine that has been shown to cause bladder cancer in humans (Melick et al., 1971). Production of the carcinogenic metabolite depends on N-hydroxylation, a process that we have shown to occur in both medaka and guppies in our studies with another aromatic amine, 2-acetylaminofluorene as described in section 7.0. In addition to using this compound to further examine the carcinogenicity of aromatic amines in small fish models, we also wished to determine whether carcinogenicity of this compound which affects only the kidney in mammals would express its carcinogenicity in another site in the fish models.

Materials and methods

This study was originally designed for both the medaka and guppy to be exposed to a single 10 ppm ABP concentration. There were three exposure groups each given a 24 hour exposure once a week for one (1 X), two (2 X), or four (4 X) weeks. A single control group was handled similarly to the 4 X exposure group except for the ABP exposure. The medaka, however, were more sensitive to the toxic effects of the 10 ppm ABP exposure than anticipated. Only about 50 of an original 100 medaka in each of the 3 treatment vessels survived the first pulse dose, which was terminated due to excessive mortality after 17 hours exposure. All surviving toxicant-exposed fish were combined into a common container, and then separated into 2 equal groups for addition to 2 growout tanks. A 1 X control group was also divided into 2 groups for assignment to growout tanks. The exposure was then redefined as a single pulse to 4-aminobiphenyl.

About 5% of the guppies in each treatment beaker died during the first pulse dose, which was prematurely terminated after 17 hours to avoid further mortality due to toxicity. To preclude the loss of additional fish to toxicity in subsequent exposures, the treatment concentration was reduced from a nominal 10 ppm to 5 ppm.

Results and Discussion

For the medaka, 20 whole specimens were examined histologically from the 1 X control group at 1 day, 18 from two 1 X ABP groups at 1 day post exposure, 10 from the 2 X ABP group at 1 week post exposure, and 8 from 4 X ABP group at 3 weeks post exposure. We detected no evidence of exposure-related toxicity.

Table 12.1 shows the results of the histopathological survey in the medaka.

Table 12.1. Incidence of hepatocellular neoplastic lesions (altered focus, adenoma and carcinoma) in the medaka (*Oryzias latipes*) exposed to 4-aminobiphenyl (ABP).

Exposure Group	13 wk	24 wk
x1 ABP	0/9	NE
x2 ABP	0/10	NE
x4 ABP	0/10	0/72
x4 Control	0/10	0/72

For the guppy, 7 whole specimens were examined histologically from the x 1 ABP group at 1 day, 17 from the x 4 control at 3 weeks, and 9 from the x 4 ABP group 3 weeks post exposure. No evidence of exposure-related toxicity was seen.

Table 12.2 shows the results of the guppy survey.

Table 12.2. Incidence of hepatocellular neoplastic lesions (altered focus, adenoma and carcinoma) in the guppy (*Poecilia reticulata*) exposed to 4-aminobiphenyl (ABP).

Exposure Group	13 wk	24 wk
x1 ABP	0/4	NE
x2 ABP	0/10	NE
x4 ABP	0/9	0/69
x4 Control	0/19	0/71

At this time, it does not appear that 4-aminobiphenyl induced neoplastic lesions in the medaka or guppy under the test conditions.

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13.0. Background neoplasms in medaka: thymic lymphoma

Introduction

Neoplasms of hematopoietic origin have been reported in several species of fish. Of these, neoplasms of the lymphoid tissues have been the most frequently reported. Epizootics of lymphosarcoma have been reported in muskellunge (*Esox masquinongy*) (Sonstegard, 1975), and in northern pike (*Esox lucius*) of North America, Ireland, Sweden, and Finland (see Thompson, 1982). Lymphosarcoma has been reported in Atlantic salmon (*Salmo salar*) (Roald and Hastein 1979) and rainbow trout (*Salmo gairdneri*) (Warr et al., 1984). Two cases of lymphosarcoma have been reported in N-methyl-N'-Nitro-N-nitrosoguanidine exposed channel catfish (*Ictalurus punctatus*) (Chen et al., 1985) and in dimethylbenz(a)anthracene exposed fish of the genus *Poeciliopsis* (Shultz and Shultz, 1982). One case of lymphosarcoma has been reported in N-methyl-N-nitrosourea treated platyfish/swordtail hybrids (Schwab et al., 1978). In addition to this, lymphoma has been reported in cultured turbot (*Scophthalmus maximus*) (Ferguson and Roberts, 1976).

The present report deals with 40 cases of lymphoma in the medaka (*Oryzias latipes*) from carcinogenesis studies conducted at the Gulf Coast Research Laboratory using this species. Twenty eight cases occurred in chemically-treated groups and 12 cases occurred in control specimens.

Materials and Methods

Medaka approximately 6-10 days old were exposed to chemical carcinogens for one to 90 days. Following exposures the fish were transferred to aquaria containing carcinogen-free water. Routine samples for histopathological examination were taken at 24, 36, and 52 weeks. Moribund fish exhibiting a swelling of the head above or near the operculum indicating the presence of lymphoma were sampled as soon as they were identified. For light microscopy, whole fish were fixed in Lillies fluid for 2-4 days, embedded in paraffin, sectioned at 5.0 µm, and were stained with hematoxylin and eosin. Moribund fish were treated in the same manner. Specimens for transmission electron microscopy (TEM) were cut from moribund fish exhibiting the tumor on the head. The tissue was fixed in 3.0% glutaraldehyde in 0.1 M sodium cacodylate buffer and then post-fixed in 1% osmium tetroxide. Specimens were then dehydrated and embedded in Embed 812 resin. Thin sections were cut using a Reichert Ultracut E ultramicrotome. Grids were stained with uranyl acetate and lead citrate and examined with a JEOL 100 SX electron microscope.

Results

The cases of thymic lymphoma observed in our medaka from cultures and carcinogenesis bioassays at the Gulf Coast Research Laboratory are listed in Table 13.1. In taking all cases as a whole, almost every tissue was infiltrated by sheets of lymphocytic cells. In early stages, lymphocytes appeared to infiltrate regions surrounding the thymus and gill arches. In more advanced stages, lymphocytic cells infiltrated the orbit, cranium, musculature, kidney, visceral peritoneum, intestine, gonads, liver, and general circulation. The basophilic nucleus with a thin rim of cytoplasm was the prominent cellular feature. Mitotic figures were abundant. The lymphocytic cell appeared to be clustered into aggregates and did not show a linear arrangement which might have suggested a lining up along reticulin fibers.

The prominent feature seen by electron microscopic examination of the lymphoma cell was a deeply clefted nucleus. Nucleoli were also prominent in the lymphoma cell as compared with normal thymic lymphocytes. The thin rim of cytoplasm contained mainly free ribosomes, mitochondria, and, frequently, centrioles.

Table 13.1. Individual cases of lymphosarcoma (thymic lymphoma; lymphoblastoma) in medaka (*Oryzias latipes*) from carcinogenicity tests conducted at the Gulf Coast Research Laboratory.

Test Group	Sex	Age
Dimethyl formamide control	ND[1]	66 wk
Control		28 wk
Puget Sound microlayer test		55 wk
Puget Sound microlayer test		55 wk
Puget Sound microlayer test		55 wk
7, 12-Dimethylbenzanthracene		40 wk
7, 12-Dimethylbenzanthracene		18 wk
Control	ND	23 wk
Dimethylformamide control		36 wk
Benzo(e)pyrene	ND	51 wk
7, 12-Dimethylbenzanthracene		34 wk
7, 12-Dimethylbenzanthracene		38 wk
7, 12-Dimethylbenzanthracene		52 wk
7, 12-Dimethylbenzanthracene	ND	21 wk
7, 12-Dimethylbenzanthracene		24 wk
Control	ND	22 wk
7, 12-Dimethylbenzanthracene/Trihalomethane mixture		24 wk
Control		52 wk

Trichloroethylene/Carbon tetrachloride		35 wk
Chlorodibromomethane		36 wk
Dibromoethane		58 wk
Dibromoethane	ND	31 wk
Dibromoethane	ND	40 wk
Dibromoethane		58 wk
Dibromoethane	ND	20 wk
Dibromoethane		35 wk
Mixture of chloroform/bromoform/bromodichloromethane		36 wk
Tributyltinoxide	ND	41 wk
Control		36 wk
Control		24 wk
Tetrachloroethane		36 wk

[1] Not Determined

Discussion

Carcinogenicity tests using small fish species have found them to be responsive to a number of chemical carcinogens (Hawkins et al., 1985). Of several species of aquarium fish used so far, the medaka (*Oryzias latipes*) has been used to study the widest range of carcinogens and the pathology has been characterized more thoroughly than for any other small species (see Metcalfe, 1989). Data on spontaneous neoplastic lesions in this species should provide useful information for evaluating results in carcinogenesis studies.

By light microscopy and transmission electron microscopy, the cells resemble lymphoma in other species including the rainbow trout TEM micrographs reported by Warr (et al., 1984). Viral particles were not observed in medaka lymphoma cells but that does not necessarily eliminate viruses in the etiology of the disease.

Medaka lymphoma reported here and in other studies differs considerably from cases reported as lymphosarcoma in carcinogen-exposed channel catfish (Chen et al., 1985) and in *Poeciliopsis* spp. (Schultz and Schultz, 1982). The catfish and *Poeciliopsis* lymphosarcomas consisted of the intense infiltration of viscera by lymphocytes which could indicate an immunologic reaction rather than a neoplasm.

In rodent bioassays it has been observed that there is a negative correlation between malignant lymphomas and hepatocellular proliferative lesions. A failure to recognize this trend may distort interpretation of carcinogenicity data for a particular test compound (Young and Gries, 1984). In our studies lymphosarcoma does not appear to affect the rate of hepatocellular lesions induced by carcinogens. This may be due to the comparatively low incidence of lymphosarcoma in medaka.

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14.0. Background neoplasms in medaka: germ cell neoplasms

Introduction

Neoplasms of reproductive tissues are generally rare in fishes and neoplasms that involve germ cells (spermatogonia and oogonia) are extremely rare (Leatherland et al., In Press). Following terminology used in human medicine (Mostofi and Price, 1973) and in fishes (Leatherland et al., In Press), seminoma and dysgerminoma are tumors of germ cells in males and females, respectively. Spermatocytic seminoma is a type of seminoma that is characterized by stages of spermatogenesis. Several germ cell neoplasms have been diagnosed in fishes including seminomas and a single case of dysgerminoma in a largemouth bass (*Micropterus salmoides*) and seminomas in Japanese dace (*Tribolodon hakonensis*) by Masahito et al. (1984). Single cases of spermatocytic seminoma have been reported in African lungfishes (*Protopterus aethiopicus*) by Masahito et al. (1984), *P. annectens* by Nigrelli and Jakowoska (1953), and *P. dolloi* by Hubbard and Fletcher (1985) and a pike eel (*Muraenesox cinereus*) by Honma (1976, cited in Leatherland et al., In Press). Down and Leatherland (1989) reported numerous cases of proliferative conditions in reproductive elements that they considered to be seminomas and spermatocytic seminomas in carp-goldfish hybrids.

Medaka (*Oryzias latipes*), widely used in carcinogenesis studies because of their carcinogen sensitivity, economy, and ease of rearing among other factors (Ishikawa et al., 1984; Hawkins et al., 1988) develops few spontaneous neoplastic lesions (Masahito et al., 1989). Only a single case of a germ cell neoplasm, an ovarian dysgerminoma, has been reported from medaka (Harada et al., 1991). Here, we report multiple cases of germ cell neoplasms that appear to be differentiating toward spermatocytic seminoma in male and female medaka that were used to test a variety of compounds for carcinogenicity.

Methods

Specimens with germ cell neoplasms described in this study came from approximately 10,000 medaka used in carcinogenesis tests and examined histologically at the Gulf Coast Research Laboratory since 1982. The original stock of orange-red variety medaka was obtained from Carolina Biological Supply Company, Burlington, North Carolina, USA. Most of the tests were carried out as follows. Medaka approximately 6- to 10-days old were exposed to test chemicals for periods that ranged from 1 hour to 180 days. Typically, a test included equal numbers of specimens assigned to an untreated control group, a solvent control group, and three or more exposure groups. Following exposure, fish were transferred to aquaria containing water free of the test chemical and fed a diet of flake food supplemented with live brine shrimp (*Artemia* sp.). Routinely, samples were taken for histopathological examination at 24, 36, and 52 weeks after the beginning of exposures or when fish became moribund. Whole fish specimens were fixed for light microscopy in Lillie's fixative (Humason, 1979) for 2 to 4 days, embedded in paraffin, sectioned at 5.0 µm, and stained with

Harris' hematoxylin and eosin. Slides of specimens were examined in two longitudinal planes.

Results

Features of the 24 cases of germ cell neoplasms in medaka form studies at the Gulf Coast Research Laboratory are summarized in Table 14.1. Ten cases occurred in phenotypic females and 12 in males. Two cases occurred in untreated control specimens. Four cases occurred in specimens exposed to compounds that were highly carcinogenic to medaka, three in specimens exposed to 7,12-dimethylbenz(a)anthracene, and one in a specimen exposed to 1,2-dibromoethane. The remaining 18 cases were in specimens exposed to compounds that were moderately, weakly, or not carcinogenic to medaka, at least under the applicable conditions of exposure concentration and time post-exposure. The average age of specimens with germ cell neoplasms was about 50 weeks. The youngest specimen in which the tumor was diagnosed was 17 weeks old and the oldest was 73 weeks. Concerning specific chemical exposures, five tumor-bearing specimens occurred in a test of a mixture of halogenated hydrocarbons, four in a test of trichloroethylene, and four in a test of 7, 12-dimethylbenz(a)anthracene. However, tumor occurrence was not related to exposure concentration of the test chemical, and, in two tests, cases occurred in control as well as exposed specimens. Most of the tumors were discovered during examination of specimens in routine sampling. Only three specimens with seminomas were moribund and only one had another type of neoplasm.

Although some of the more advanced tumors caused abdominal swelling that could be observed grossly, the cases described here were seen histologically; no material was available for ultrastructural examination.

Most of the germ cell neoplasms were confined to the abdominal cavity. In males, the neoplasms appeared to originate near the normal location of the testis. Some of the tumors were as large as 3.5 x 5.0 mm and nearly filled the abdominal cavity. The tumors in males exhibited two principal cellular patterns, lobular and solid. The stroma of the tumors was sparse and there was no evidence of lymphocytic infiltration or granuloma formation. In most tumors with a lobular pattern, cells resembled primary spermatocytes, secondary spermatocytes, and spermatids. None of these neoplasms, however, contained mature spermatozoa. Mitoses were no more abundant than would be expected in normal testis. For each cell type, chromatin appeared to be normally distributed. Tumors with the solid pattern consisted mainly of cells resembling primary spermatocytes and spermatids. The neoplasms also contained small dense cells that appeared to be degenerating primary spermatocytes.

Table 14.1. Germ cell neoplasms in medaka *Oryzias latipes* used in carcinogenesis tests at the Gulf Coast Research Laboratory.

Case	Sex	Test Group	Exposure Period	Age	Histologic Features
1	M	0.5 ppm DMF	6 hrs x 2	17 wk	lobular; oocytes
2	F	0.2 ppm THM	90 days	36 wk	cord-like
3	M	0.5 ppm DMF	6 hrs x 2	36 wk	lobular; oocytes
4	M	Control	-	52 wk	lobular; no oocytes
5	M	2.0 ppm THM	90 days	52 wk	lobular; oocytes
6	M	0.2 ppm THM	90 days	52 wk	lobular; early (?)
7	F	2.0 ppm THM	90 days	52 wk	solid/cord-like
8	M	15 ppb DMBA	6 hrs x 3	60 wk	solid/lobular; no oocytes
9	F	Control	-	60 wk	cord-like
10	F	15 ppm CDBM/CCl_4	90 days	36 wk	lobular
11	F	0.1 ppm TCE	90 days	53 wk	lobular
12	F	15 ppm TCE	90 days	53 wk	cord-like
13	M	15 ppm TCE/CCl_4	90 days	53 wk	lobular; no oocytes
14	M	20 ppm DBE	90 days	36 wk	lobular; oocytes
15	M	0.1 ppm TCE	90 days	66 wk	cord-like/solid; oocytes
16	F	30% PSSM	6 hr	36 wk	cord-like
17	M	75 ppb BeP/DMF	6 hr x 2	24 wk	lobular; oocytes
18	M	30% PSSM	6 hr	55 wk	lobular; oocytes
19	M	0.5 ppm DMF	6 hr	36 wk	lobular; oocytes
20	F	61 ppb DMBA	6 hr	52 wk	lobular
21	F	60 ppm Cd	24 hr x 4	38 wk	lobular
22	F	15 ppm TeCe	90 days	53 wk	multiple
23	M	51 ppb DMBA	6 hr	73 wk	lobular; no oocytes
24	M	1 ppm AAF	12 hr x 2	52 wk	solid; oocytes

Abbreviations used in Table 14.1: DMF=dimethylformamide; THM=mixture of halogenated hydrocarbons containing carbon tetrachloride, bromoform, chloroform, chlorodibromomethane, bromodichloromethane, and trichloroethylene; DMBA=7,12-dimethylbenz(a)anthracene; CDBM=chlorodibromomethane; CCl4=carbon tetrachloride; TCE=trichloroethylene; DBE=ethylene dibromide (1,2-dibromoethane); PSSM=Pudget Sound sea surface microlayer fraction; Cd=cadmium; TeCE=1,1,2,2-tetrachloroethane; BeP= benzo(e)pyrene; AAF= 2-acetylaminofluorene (N-2-fluorenylacetamide).

Germ cell neoplasms in 8 of the 14 tumor-bearing male specimens contained large cells resembling oocytes that usually were scattered individually throughout the tumors but sometimes were abundant and occurred in clusters. The oocyte-like cells were characterized by a dense cytoplasm and a large clear or vesiculated nucleus. In some of the larger oocyte-like cells, small round structures that resembled nucleoli of maturing oocytes lined the nuclear membrane and a structure resembling a vesicular ring lay outside the nuclear membrane.

Germ cell neoplasms in female specimens appeared to arise within ovaries, usually at the periphery beneath the ovarian wall, and appeared as large compact or loosely dispersed masses. Most of the neoplasms in females exhibited a cord-like pattern consisting of cells that resembled primary spermatocytes and spermatids. Others, however, had solid or lobular cellular patterns in which the tumor cells resembled primary spermatocytes. Unlike the germ cell neoplasms in males, those in females did not have oocyte-like cells in the tumor masses.

Although the germ cell neoplasms did not often appear to cause the deaths of affected specimens, several were locally invasive and some appeared to have metastasized. Germ cell neoplasms in females showed a greater tendency to be invasive than those in males. In one specimen, two major tumor masses occurred in a female specimen, and the posterior mass invaded through the abdominal peritoneum into body wall musculature. Invasion of the peritoneum was well advanced in some cases, and tumor tissue extended anterior to the liver and into the pericardial cavity. Invasion of the peritoneal lining occurred in which neoplastic cells lie on both sides of the lining. That tumor invaded and replaced much of the kidney.

Discussion

Epizootics of gonadal neoplasms have been reported in hybrids of carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*) from the Great Lakes (Sonstegard, 1978; Down and Leatherland, 1989) and multiple cases of ovarian neoplasms have been described in domestic and ornamental carp (Ishikawa et al., 1976, 1977). Gonadal neoplasms similar to those described in the present study have not been previously described in medaka. Even the single case of ovarian dysgerminoma described by Harada et al. (1991) did not histologically resemble the germ cell neoplasms described in the present paper.

Human spermatocytic seminoma is characterized by three cell types that include medium-sized round cells with eosinophilic cytoplasm that constitute the major cellular population, cells resembling secondary spermatocytes, and large mononuclear cells (Mostofi and Price, 1973). The diagnosis of spermatocytic seminoma to describe lesions could be justified by the fact that the neoplasms histologically exhibit stages of spermatogenesis. Furthermore, that diagnosis appears to hold for lesions seen in female as well as in male specimens. This contrasts with neoplasms of primordial germ cells (spermatogonia in males and oogonia in females) which are termed typical seminoma in the male and dysgerminoma in the female (Mostofi and Price, 1973). The apparent differentiation of secondary spermatocytes to spermatids in humans is evidenced ultrastructurally.

The large size of some of the tumors relative to the size of affected medaka as well as the propensity of the tumors to metastasize, would suggest some degree of malignancy for the condition. Of the 24 specimens with spermatocytic seminoma examined for this study, however, only three were found in moribund specimens. The remaining 21 specimens were collected as routinely scheduled samples. Similar behavioral features were exhibited by the medaka dysgerminoma. This tumor was also locally invasive, infiltrating the thyroid gland and between abdominal muscle bundles (Harada et al., 1991).

The present study did not identify an etiological agent for the germ cell neoplasms in medaka. The pattern of occurrence of the medaka lesions along with analyses of epizootiological studies all suggest that the lesions are not chemically induced. Wester and Canton (1986) reported the occurrence of oocytes in medaka testes of specimens exposed to ß-hexachlorocyclohexane. This is unusual because spontaneous intersexuality in medaka is rare (Yamamoto, 1969). Ovotestis has also been induced in medaka by treatment with several estrogens (Okada, 1964; Yamamoto, 1969). Although neoplasms have been reported from nearly every fish tissue, only epizootics of hepatic and epidermal neoplasms in fishes are highly correlated with environmental contamination (Harshbarger and Clark, 1990).

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15.0. Background neoplasms in medaka: pancreatic acinar cell carcinoma

Introduction

Neoplasms of the exocrine pancreas are uncommon in wild fishes and have been reported mainly as isolated cases in diverse species (Fournie et al., 1988; Thiyagarajah and Bender, 1988; Fournie and Hawkins, In Press). Exocrine pancreatic neoplasia, however, is readily induced in the guppy (*Poecilia reticulata*) by exposure to methylazoxymethanol acetate, a direct-acting carcinogen (Fournie et al., 1987). Also, a Gulf killifish (*Fundulus grandis*) injected with N-methyl-N'-nitro-N-nitrosoguanidine as an embryo developed a pancreatic acinar cell carcinoma (Grizzle et al., 1988). Thiyagarajah and Grizzle (1986) described pancreatic neoplasms of duct cell origin induced by diethylnitrosamine in rivulus (*Rivulus marmoratus*).

This report concerns the occurrence of exocrine pancreatic carcinoma in specimens of the medaka (*Oryzias latipes*) used in carcinogenesis tests. The medaka is sensitive to the hepatocarcinogenic effects of compounds representing several classes of carcinogens including nitrosamines, azoxy compounds, and mycotoxins (see review by Couch and Harshbarger, 1985) and to benzo(a)pyrene (Hawkins et al., 1988b), 7,12-dimethylbenz(a)pyrene (Hawkins et al., 1990), and 1,2-dibromoethane (unpublished). Few studies on background neoplasms in medaka have been reported. Masahito et al. (1989) reported a low incidence of hepatic neoplasms in medaka older than one year and the rare occurrence of extrahepatic neoplasms that included squamous cell carcinoma, melanoma and lymphosarcoma. Torikata et al. (1989) described a case of spontaneous olfactory neuroepithelioma in a medaka. The spontaneous occurrence of thymic lymphoma (lymphoblastic lymphoma or lymphosarcoma) has also been reported (Battalora et al., 1990; Okihiro and Hinton, 1989; Harada et al., 1990). Here, we examined the occurrence and pathogenesis of exocrine pancreatic carcinoma presumably of acinar cell origin in the medaka.

Methods

Specimens examined in this study came from carcinogenesis tests conducted on medaka (*Oryzias latipes*). The original stock of orange-red variety medaka were obtained from Carolina Biological Supply Company, Burlington, North Carolina, USA, and have been cultured in our laboratory since 1982. The bioassays were carried out generally as follows. Medaka approximately 6 to 10 days old were exposed to a variety of test chemicals for times that ranged from 1 hour to 180 days. Typically, a test included equal numbers of specimens assigned to an untreated control group, a solvent control group, and at least three exposure groups. Following exposure, fish were transferred to aquaria containing carcinogen-free water and fed a diet consisting of flake food (Enriched Stress Flakes and Maintenance Flakes, Novalek, Inc., Hayward, CA) supplemented with brine shrimp nauplii (*Artemia* sp., Product No. 84-8990, Novalek, Inc., Hayward, CA and Product BS-3, lot 756, Aquarium Products, Glen Burnie, MD). Routinely, samples were taken for histopathological examination at 24, 36, and 52 weeks after the beginning of the exposures or when they became moribund.

Whole fish specimens were fixed for light microscopy in Lillie's fixative (Humason, 1979) for 2 to 4 days, embedded in paraffin, sectioned at 5.0 µm, and stained with Harris' hematoxylin and eosin. Slides of specimens were examined in two longitudinal planes of section. Culture, exposure, and test conditions are described above in detail in section *3.0. Materials and Methods.*

Results

The cases of exocrine pancreatic carcinoma in medaka are summarized in Table 15.1. Eight cases were diagnosed from approximately 10,000 specimens older than 24 weeks used in carcinogenesis tests for an overall incidence of 0.08%. About one-fourth of the total number of specimens were untreated controls. Considering the small size of adult medaka (about 25 mm standard length) with exocrine pancreatic carcinoma, some tumors were quite large, attaining a maximum of 2.5 mm. Of the eight medaka with tumors, six were female and two were male. The average age of the tumor-bearing specimens was about 48 weeks. The youngest specimen diagnosed with exocrine pancreatic carcinoma was 24 weeks and the oldest was 78 weeks. Lesions occurred in the following cases: (1) a 36-week-old female exposed to MAM-Ac; (2) a 44-week-old female control specimen; (3) a 73-week-old female control specimen; (4) a 25-week-old female control specimen; (5) a 33 week old female exposed to DMBA; (6) a 42 week old male exposed to BaP; (7) a 43-week-old male exposed to BeP; and, (8) a 78-week-old female exposed to DMBA. Five of the eight cases were diagnosed in moribund specimens in which the tumor appeared to be the cause of death. In three cases, tumors were found in specimens that were examined as part of routinely scheduled samples.

Based on anatomic location, cellular architecture, and malignant behavior, each case could be described as poorly differentiated acinar cell carcinoma. The tumors apparently originated from pancreas in the mesentery near the liver. Some of the tumors were extensive and exhibited aggressive growth patterns. Most of the tumors infiltrated the peritoneal cavity and sometimes invaded the gonads and intestine. Tumor cells also invaded the peritoneum and infiltrated retroperitoneal spaces, the kidney, and spaces in the head region. The presence of tumor emboli in the cardiac ventricle and tumor cells attached to trabeculae of the atrial myocardium suggested that some tumors spread through the blood.

Histologically, the tumors consisted primarily of poorly differentiated basophilic cells that tended to form into lobules or cords. Many of the cells exhibited cellular pleomorphism, nuclear atypia, a high nucleus-to-cytoplasm ratio, and an elevated mitotic index. Tumor cells showed no evidence of gland or tubule formation. Some tumors, however, contained neoplastic cells with granular eosinophilic inclusions. These cells were probably well-differentiated exocrine pancreatic cells filled with zymogen granules. Central portions of the tumors often consisted of poorly differentiated, spindle-shaped cells that contained little or no zymogen. Areas of necrosis were also frequently seen near the centers of the larger tumors.

Table 15.1.- Features of exocrine pancreatic carcinomas in medaka (*Oryzias latipes*) from carcinogenesis tests conducted at the Gulf Coast Research Laboratory.

Case No.	Sex	Test Group	Duration of Exposure	Age
1	F	MAM	2 hr	36 wk
2	F	Control	-	44 wk
3	F	Control	-	73 wk
4	F	Control	-	25 wk
5	M	DMBA	6 hr	33 wk
6	M	BaP	2 x 6 hr	42 wk
7	M	BeP	2 x 6 hr	43 wk
8	F	DMBA	6 hr	78 wk

Abbreviations: F-Female; M-Male; MAM-Methylazoxymethanol acetate; DMBA- 7,12-Dimethylbenz(a)anthracene; BaP-Benzo(a)pyrene; BeP-Benzo(e)pyrene; wk-Week; * Moribund specimen

Discussion

Four of the eight cases of exocrine pancreatic carcinoma occurred in medaka exposed to compounds that also induced other types of neoplastic lesions in that species. One case was in a specimen exposed to MAM-Ac, a direct-acting carcinogen that induces a variety of neoplastic lesions in the medaka following a single, brief exposure (Hawkins et al., 1988a). Two cases occurred in specimens exposed to DMBA which also causes neoplasms in several different organs in medaka (unpublished), and a single case in a specimen exposed to BaP which causes hepatic neoplasms in medaka (Hawkins et al., 1988b). Three cases occurred in designated control specimens and one case in a medaka exposed to BeP, a non-carcinogenic isomer of BaP. Based on the occurrence of these tumors in control specimens and the fact that carcinogen exposure did not cause a concentration-related increase in the incidence of the tumors, we consider pancreatic acinar cell carcinoma in medaka to be spontaneous and probably not induced by exposure to carcinogens, at least not by the carcinogens and protocols tested here. Most carcinogens tested in fish models target hepatocellular and cholangiocytic elements of the liver (Couch and Harshbarger, 1985; Hatanaka et al., 1982; Hendricks et al., 1984). Thorough assessment of the carcinogenicity of a test compound, however, depends on understanding the occurrence and pathogenesis of hepatic and nonhepatic spontaneous neoplasms as well as rarely-occurring, carcinogen-induced neoplasms.

Thymic, or lymphoblastic, lymphoma (Battalora et al., 1990; Okihiro and Hinton, 1989; Masahito et al., 1989; Harada et al., 1990) and exocrine pancreatic carcinoma (this study) appear to be spontaneous neoplasms in medaka whereas several other rarely-occurring neoplasms including various sarcomas, neural, and kidney neoplasms apparently can be carcinogen-induced (Hawkins et al., 1988a; unpublished).

The overall low incidence of exocrine pancreatic neoplasms in medaka contrasts with the high sensitivity of the guppy exocrine pancreas to the carcinogenic effects of MAM-Ac (Fournie et al., 1987). Under identical exposure conditions, MAM-Ac induced exocrine pancreatic neoplasms in the guppy but failed to induce them in six small fish species including the medaka. In that study, each species developed hepatocellular tumors following MAM-Ac exposure (Hawkins et al., 1988a). The MAM-Ac-induced acinar cell carcinomas in the guppy exhibited several cellular patterns, but were for the most part rather well-differentiated neoplasms (Fournie et al., 1987). Additionally, the guppy pancreatic carcinomas were only locally invasive and showed no evidence of metastasis. The medaka pancreatic carcinomas, on the other hand, were poorly differentiated, aggressive neoplasms as evidenced by their size, elevated mitotic activity, growth pattern, and metastatic spread. The medaka tumors were also histologically similar to the poorly differentiated acinar cell carcinomas and undifferentiated pancreatic carcinomas in azaserine-treated rats (Longnecker et al., 1981). Multiple hepatic metastasis and regional lymph node involvement were also observed in these rats.

There are several reasons for studying the occurrence and biology of spontaneous and rare neoplasms in small fish carcinogenesis models. In developing carcinogenesis bioassays, one needs to know as much as possible about the capability of the model to develop any kind of neoplastic lesion, whether spontaneous or chemically-induced. Furthermore, the carcinogenic potency and target organs of a test compound could be misinterpreted if the historical incidence of rare extrahepatic neoplasms is not known or considered.

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16.0. Background neoplasms in the guppy: adenocarcinoma of the retinal pigment epithelium in the guppy

Introduction

Neoplasms involving optic tissues have been rarely reported in fishes. The Registry of Tumors in Lower Animals (RTLA) at the National Museum of Natural History, Washington, D.C. contains only nine cases (Harshbarger, 1991). Accessions include both published and unpublished cases. Dawe and Harshbarger (1975) illustrated a retinoblastoma in a croaker (RTLA 650), Fournie and Overstreet (1985) reported a retinoblastoma in a spring cavefish (*Chologaster agazzi*, RTLA 2901), Reimschuessel et al. (1989) reported retinoblastomas in a brown bullhead (*Ictalurus nebulosus*, RTLA 3971) and a porkfish (*Anisotremus virginicus*, RTLA 3972), and Courtney and Fournie (1991) reported two cases of ocular chondrosarcoma in rivulus (*Rivulus marmoratus*, RTLA 3973 and 3974). The two unpublished cases are retinoblastomas in green swordtails (*Xiphophorus helleri*, RTLA 2030 and 2747). Other reported ocular tumors not accessioned in the RTLA include five cases of melanoma from *Platypoecilus maculatus*, *Xiphophorus pygmaeus*, and *P. maculatus* - *X. helleri* hybrids (Levine and Gordon, 1946) and a single case of a ciliary body medulloepithelioma from a goldfish, *Carassius auratus* (Lahav and Albert, 1978). There is only one report of experimentally-induced ocular neoplasms in fishes. Hawkins et al. (1986) described the occurrence and development of medulloepitheliomas in medaka following a single brief exposure to the direct acting carcinogen methylazoxymethanol acetate (MAM-Ac). In this paper, we describe a retinal pigment epithelium adenocarcinoma from a guppy (*Poecilia reticulata*, RTLA 3139), the first such neoplasm reported from fishes.

Materials and Methods

The affected fish was from a carcinogen bioassay in which several hundred specimens were exposed to a trihalomethane (THM) mixture plus phenol. The exposure regime consisted of a single 24 hour pulse dose with 40 ppm THM followed by four 24 hour pulse doses (all 7 days apart) with 15 ppm phenol. The specimen was sampled 312 days post exposure, fixed in Lillie's solution, decapitated, and the separate body parts were processed and embedded in paraffin. Sections of the head were cut in the coronal plane and sections of the body were in the parasagittal plane. Histologic sections were all 6 μm thick and were stained with Harris' hematoxylin and eosin or alcian blue.

Results

Only one of the exposed specimens exhibited an ocular neoplasm and none of the control specimens in this study exhibited any neoplastic lesions. The left eye of the affected fish was distended to over twice its normal diameter by a tumor mass occupying the area of the vitreous body. The tumor consisted mainly of bilayered, serpentine melanin containing epithelial tissue separated by a loose fibrovascular stroma. Each layer consisted of a single row of columnar epithelium containing varying amounts of pigment. In some areas, the proliferating cells were arranged in solid sheets and did not produce the tubular pattern. Tumor cells in these areas contained less pigment and exhibited extensive cellular pleomorphism and nuclear atypia. Heavily pigmented aggregates of cells which occurred in several areas of the tumor were probably macrophages with phagocytosed pigment released from the tumor cells. Cornea, annular ligament, and the anterior chamber were not affected, however, tumor was attached to the iris and large portions of the choroid were destroyed. Tumor extended to, but not through, the sclera and tumor cells occurred at the periphery of the optic nerve at its insertion into the globe.

Discussion

This tumor apparently originated from retinal pigment epithelium rather than choroidal pigment cells, in view of the distinct epithelial appearance of the tumor cells, the tendency of the tumor cells to palisade as in the retina, and the extreme anterior displacement of the retina by the tumor. Additionally, the distinct tubular growth pattern exhibited by this tumor is characteristic of human retinal pigment epithelium adenomas and adenocarcinomas (Hogan and Zimmerman, 1962).

The guppy, as well as some other small fish species, apparently do not readily develop intraocular neoplasms after carcinogen exposure. In studies designed to find suitable fish models for long-term testing of low doses of waterborne carcinogens, Hawkins et al. (1988) exposed young specimens of seven small fish species, including the guppy, to a single dose of MAM-Ac to determine their susceptibility to that recognized carcinogen. The medaka was the only species to develop intraocular neoplasms and 45% of the exposed fish examined had neoplastic lesions in various stages of development (Hawkins et al., 1986). The apparent insensitivity of the guppy retina to neoplastic change was further demonstrated in this study in which only a single case of retinal pigment epithelium adenocarcinoma occurred.

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17.0. Spontaneous hepatic proliferative lesions in designated control specimens of medaka and guppy from carcinogenicity tests at the Gulf Coast Research Laboratory

Introduction

Several small fish species including the medaka (*Oryzias latipes*) and the guppy (*Poecilia reticulata*) are considered primary candidates for carcinogenesis bioassay models (Ishikawa et al., 1984; Zimmerer, 1984; Hawkins et al., 1988a). One of the attributes of the small fish species is the low incidence of spontaneous neoplasms in target organs of control specimens (Dawe and Couch, 1984). Correspondingly, few spontaneous neoplasms of the liver, the most frequent site of neoplasia following carcinogen exposure, or from other organs in those species have been reported (Masahito et al., 1989). Here, we report the incidences of hepatocellular neoplastic lesions (altered foci, adenomas and carcinomas) and of biliary neoplasms (cholangiomas and cholangiocarcinomas) in control specimens of medaka and guppy from carcinogenesis bioassays. Although the diagnostic criteria used here have been applied in other studies on the carcinogenic responses of small fish to several carcinogenic compounds (Hawkins et al. 1988, 1989, 1990), a conventional scheme of diagnosis and nomenclature has not been firmly established and accepted for neoplastic lesions in small fish.

Methods

History of the fish colonies. Specimens examined in this study came from carcinogenesis tests conducted on medaka and guppy in our laboratory. The original stock of orange-red variety medaka was obtained from Carolina Biological Supply Company, Burlington, North Carolina, USA, and have been cultured in our laboratory since 1984. Guppies were the king cobra strain originally purchased as a lot of 200 fish in 1982 from a commercial pet dealer, and broodstock was supplemented from the same source in 1984. Details of bioassay protocols, dietary regimes and histopathological methodology are found above in section *3.0. Materials and Methods.*

Bioassay protocols. The bioassays were carried out generally as follows. Medaka and guppies approximately 6- to 10- days old were exposed to a variety of test chemicals for times that ranged from 1 hour to 180 days. Control groups for those tests generally were either static (aquarium) controls, flow-through control, or solvent controls. Following exposure, fish were transferred to aquaria containing carcinogen-free water. Fish were exposed and maintained in 27 ± 1 °C.

Diet. Juvenile and adult fish were fed a diet consisting of a commercially produced flake food supplemented with live brine shrimp (*Artemia* sp.).

Histopathological examination. Routinely, samples were taken for histopathological examination at 24, 36, and 52 weeks after the beginning of the exposures. In other words, sampling times were approximately equal to the age of the specimens. For light microscopy, whole fish were fixed in Lillie's solution for 2 to 4 days, embedded in paraffin, sectioned at 5 μm, and stained with Harris' hematoxylin and eosin. Typically, slides of specimens were examined in two longitudinal planes of section, one about mid-sagittal and the other near the midline.

Results

Occurrence. Tables 17.1 and 17.2 show the occurrence of hepatic proliferative lesions in medaka and guppy, respectively. Lesions accounted for included hepatocellular altered foci, hepatocellular adenoma, hepatocellular carcinoma, cholangioma, and cholangiocarcinoma for the three sampling periods.

Diagnostic criteria. Table 17.3 lists the diagnostic criteria used in this study for the hepatocellular lesions.

Discussion

The occurrence of spontaneous hepatocellular and biliary proliferative lesions in the medaka (*Oryzias latipes*) and guppy (*Poecilia reticulata*) is reported. Data represent approximately 4000 medaka and 1000 guppies used as designated control specimens in various carcinogenesis bioassays. Diagnoses were made from hematoxylin and eosin stained sections of specimens examined at approximately 24, 36, and 52 weeks of age for the presence of hepatocellular lesions including foci of cellular (staining) alteration (altered foci), adenoma, and carcinoma and biliary lesions including cholangioma and cholangiocarcinoma. Incidences of the hepatocellular lesions were low for both species. For example, the highest incidence recorded for medaka was 0.16% (3/1877) for altered foci from the 36-week sample and for guppy was 1.3% for carcinomas in the 36-week sample. Similarly, biliary neoplastic lesions were also low, with none being diagnosed in any guppy specimens. The low number of bioassay-relevant spontaneous neoplastic lesions in control medaka and guppy substantiates the statistical power of the small fish carcinogenesis tests.

Table 17.1. Spontaneous hepatic proliferative lesions in control medaka (*Oryzias latipes*) from the Gulf Coast Research Laboratory.

	24 Week (N=1815)	36 Week (N=2193)	52 Week (N=939)
Hepatocellular			
Altered Focus	2 (0.1%)	0	0
Adenoma	0	4 (0.2%)	2 (0.2%)
Carcinoma	0	3 (0.1%)	1 (0.1%)
Total	2 (0.1%)	7 (0.3%)	3 (0.3%)
Cholangiolar			
Cholangioma	0	0	0
Cholangiocarcinoma	0	0	0
Total	0 (0%)	0 (0%)	0 (0%)

Table 17.2. Spontaneous hepatic proliferative lesions in control guppies (*Poecilia reticulata*).

	24 Week (N=1035)	36 Week (N=1240)	52 Week (N=555)
Hepatocellular			
Altered Focus	0	3 (0.2%)	4 (0.7%)
Adenoma	0	2 (0.2%)	1 (0.2%)
Carcinoma	0	1 (0.1%)	2 (0.4%)
Total	0 (0%)	6 (0.5%)	7 (1.3%)
Cholangiolar			
Cholangioma	0	0	0
Cholangiocarcinoma	0	0	0
Total	0 (0%)	0 (0%)	0 (0%)

Table 17.3. Diagnostic criteria for hepatocellular lesions in medaka and guppies in hematoxylin and eosin stained sections.

Altered focus

-eosinophilic or basophilic
-tends to blend with surrounding normal tissue at periphery of lesion
-little or no disruption of parenchymal pattern in focus
-cells retain ability to store glycogen and/or fat similar to that in surrounding normal hepatocytes
-mitotic figures rare
-no cellular pleomorphism
-focus contains other hepatic cell types
-profiles usually uniformly round in section

Adenoma

-usually basophilic
-cells distinct from surrounding normal tissue
-profiles usually uniformly round
-mitotic figures occasionally seen
-hepatocytes tend to be pleomorphic (either larger or smaller than normal)
-hepatocytes unable to store glycogen and/or lipid to same extent as normal tissues
-parenchymal pattern in lesion distinct from that in surrounding tissue but usually only two cells thick
-lesions composed principally of hepatocytes

Carcinoma

-lesions basophilic
-cells at periphery of lesion invade and/or compress adjacent normal tissues
-borders are usually irregular but lesion is distinct from surrounding tissues
-lesion profiles usually irregular
-lesions usually occupy about 25% or more of liver
-hepatocytes store no glycogen or fat
-mitoses easily found in lesion
-lesions consist solely of hepatocytes
-lesion may contain areas of dedifferentiated cells
-cells and their nuclei are pleomorphic
-if parenchymal pattern evident, cords (or tubules) are thickened

Clearer diagnostic criteria exist between altered foci in control medaka and guppies and adenoma than between adenoma and carcinoma. The lack of agreement in nomenclature is shown in at least two other studies (Masahito et al., 1989 and Harada et al. 1988) in which lesions similar to those altered foci were described as adenoma. This study substantiates the low rate of occurrence of altered foci in control medaka and guppies. In Fisher 344 rats used in carcinogenesis bioassays the spontaneous incidence of foci of cellular alteration is 80% by 9 months and 100% by 15 months (Maronpot et al., 1989). Ample justification thus exists for classifying the lesions described in this study as altered foci as adenomas and combining adenomas with carcinomas. This would assume that the originally-diagnosed adenomas were early stages in the development of carcinomas.

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18.0. Personnel

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19.0. Publications

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